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EFFECT OF HOT SOLUTIONS OF SULFOSALICYLIC ACID ON VARIOUS PROTEINS

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As is known, solutions of sulfosalicylic acid are used as protein precipitants; a 20% sulfosalicylic acid solution finds wide use in clinical laboratories as a reagent in protein (albumin) detection or to check the completeness of protein precipitation.

There are literature references to the effect that precipitates which form in the cold upon addition of sulfosalicylic acid are characterized by their insolubility upon heating the acid suspension obtained, and that in contrast, similar sulfosalicylic precipitates formed from so-called albumoses or proteoses (protein nondialyzable products with a molecular weight of 10,000 to 30,000) dissolve upon heating [1]. Based on this difference in solubility upon heating of the sulfosalicylic acid precipitates, some authors proposed a possible method for differentiating proteins from proteoses. This method is used in clinical laboratories for the quantitative determination of proteoses of the blood in different pathological conditions [2].

In using the indicated method for determination of proteoses, we observed that the description of the insolubility of the sulfosalicylic acid protein precipitates upon their being heated together with the solution from which they precipitated did not correspond to the facts. Thus these methods for separation of proteoses from proteins appeared to be invalid. In this connection, and also so that studies which might be undertaken in this area would receive the proper interpretation, it seemed to us expedient to publish the data obtained.

I. Precipitation of γ-Globulin by Sulfosalicylic Acid Solutions

In these tests an ampule solution of γ-globulin which was produced as an antimeasles preparation, with a content of ~ 8% γ-globulin, was used.

The γ-globulin solution was treated as follows. 2.5 ml was pipetted into a flask containing 235 ml of distilled water. The mixture was stirred and precipitated with 12.5 ml of 20% sulfosalicylic acid. There was finally obtained 250 ml of a solution in which the total protein concentration was ~ 0.08% and the sulfosalicylic acid concentration 1%, approximately 0.04 M, of pH 1.6.

After the solution was shaken, an abundant precipitate of protein formed. The suspension was then heated to boiling. Upon application of heat, there was a considerable lightening of the liquid, and during boiling a large portion of the precipitate redissolved.

Thus, in the γ-globulin solution, proteins which dissolved in a hot solution of sulfosalicylic acid were undoubtedly present.

Upon filtration of the hot liquid through a hot water funnel, only an insignificant precipitate remained on the filter; the transparent filtrate comprised a solution of the main portion of the γ-globulin in an excess of sulfosalicylic acid. When the solution cooled, an abundant precipitate formed.

In order to establish a quantitative relationship between the soluble and insoluble portions of protein upon heating, we carried out a nitrogen determination by the Kjeldahl method in Conway vessels in the following fractions:

1) in the γ-globulin solution taken directly from the ampule (total nitrogen);

2) in the hot sulfosalicylic acid filtrate obtained after separation of the protein insoluble after heating (nitrogen soluble in hot sulfosalicylic acid);

3) in the cold sulfosalicylic acid filtrate, obtained after filtration of the protein which precipitated from the cold solution, that is, nitrogen-soluble in cold sulfosalicylic acid.

As indicated in Table 1, the concentration of protein contained in the ampules averaged the anticipated 8% (7.7-9.3%). Unexpectedly, the hot sulfosalicylic acid solution dissolved almost all the protein: in one test 88 and in another (within the limits of error of the method) even 100%.

TABLE 1

Some Nitrogen Fractions in Solutions of Protein Preparations

Preparation	Total nitrogen in original solution		Nitrogen soluble in hot sulfosalicylic acid		Nitrogen soluble in cold sulfosalicylic acid	
	mg%	calculated as % protein	mg%	% of total nitrogen	mg%	% nitrogen in hot sulfosalicylic acid
γ-Globulin	1482	9.3	1484	100	102	7
"	1237	7.7	1092	88	47	4
Hemoglobin	630	3.9	546	87	99	18
Serum albumin	933	5.8	70	7.6	28	40
" "	945	5.9	56	5.9	33	60

Naturally, it cannot be assumed that the γ-globulin solution consisted almost entirely of substances resembling proteoses or albumoses. Thus, the quantitative determination confirmed the previous conclustion that γ-globulin as such was soluble in hot sulfosalicylic acid solution.

The analysis showed further that in the hot sulfosalicylic acid solution of protein, there were low molecular weight nitrogenous substances of nonprotein character, to which, naturally, belonged substances which remain in solution after protein precipitation upon its cooling. These substances were present in amounts from 47 to 102 mg%, that is, comprising 4-7% nitrogen from the hot sulfosalicylic acid solution. Two assumptions can be made as to the origin of these substances: either they were present from the very beginning in the ampule, or they formed during the process of heating the protein in the sulfosalicylic acid solution.

II. Precipitation of Hemoglobin by Sulfosalicylic Acid

We decided further to study the effect of hot sulfosalicylic acid on hemoglobin* solutions.

The hemoglobin solution (about 4%) was precipitated by sulfosalicylic acid under the conditions described for γ-globulin, with a final protein concentration of 0.08%, and that of sulfosalicylic acid 1% (0.2 ml 4% Hb solution + 9.3 ml H_2O + 0.5 ml 20% sulfosalicylic acid). The colorless solution with the brownish-red protein precipitate obtained was heated to boiling, whereupon it was observed that the major portion of the precipitate redissolved. The hot filtered solution had a red color and upon cooling a dark brown precipitate formed.

A quantitative nitrogen determination was carried out on the same fractions as for γ-globulin.

As seen from Table 1, 87% of the protein (hemoglobin) present in the original solution was found to redissolve in the hot sulfosalicylic acid. A large portion of this quantity again precipitated upon cooling of the solution, and only 18% of the protein (99 mg%) remained in the filtrate obtained after cooling the hot solution.

From these data it can be concluded that the hemoglobin as well as the γ-globulin interacted with the sulfosalicylic acid, forming compounds which almost completely dissolve in hot solutions of this acid. Upon cooling, protein compounds with sulfosalicylic acid reprecipitate.

* The hemoglobin preparation, as in the case of the serum albumin, which will be discussed later, was isolated from ox blood in the protein fractionation laboratory and kindly made available to us by G. Ia. Rozenberg, to whom we express our gratitude.

III. Precipitation of Albumin Solutions by Sulfosalicylic Acid

The same studies were carried out on 6% solutions of purified serum albumin (precipitation conditions the same as for γ-globulin). In this case the findings were different. It was noted right away that the sulfosalicylic precipitate of the albumin compounds did not change upon heating or even upon boiling the solution. The solution filtered through a hot-water funnel with much difficulty, since the filter was filled with protein precipitate.

Quantitative determination of nitrogen in the fractions (in two tests) yielded results differing markedly from those described for γ-globulin and hemoglobin.

As can be seen from Table 1, only 5.9-7.6% of the protein formerly in solution (actual nitrogen) was soluble in hot sulfosalicylic acid solution. If it is to be borne in mind that of this quantity of nitrogen some portion, namely 28-33 mg% (i. e., approximately half) comes from nitrogen remaining in solution after cooling of the liquid, then it is easy to estimate that in the case of the albumin, the portion of true protein nitrogen capable of dissolving in hot sulfosalicylic acid is only 3-4% of the entire albumin preparation taken.

Electrophoretic Investigation

The question arose as to whether during solution of the protein in hot sulfosalicylic acid, the original integrity of the protein molecule was preserved or whether the molecule underwent considerable change under the influence of heating with the acid. In such a case, the phenomenon of hot solution was not essentially inherent in the protein molecule as such, but was related to the disintegration (hydrolysis) into smaller fractions.

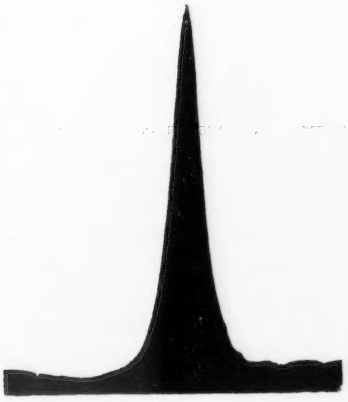

Fig. 1. Electrophoregram of γ-globulin after treatment with hot sulfosalicylic acid. N concentration – 126 mg%. Descending arm, 211 min after beginning of electrophoresis; curent : 8 ma, 80 v.

Fig. 2. Electrophoregram of the mixture: preparation of γ-globulin after treatment with hot sulfosalicylic acid, original γ-globulin and serum albumin. Descending arm, 188 min after the beginning of electrophoresis; flow: 8 ma, 80 v. Peak A) albumin; B') treated γ-globulin; B) γ-globulin.

It was hoped that comparative electrophoresis of the original protein solution and preparations obtained from the same proteins after the described processing with sulfosalicylic acid would afford an answer to this question.

The investigations were conducted as follows.

The precipitate which resulted from cooling of the hot sulfosalicylic acid, representing, in all likelihood, compounds of protein with sulfosalicylic acid, was filtered off and dissolved in a minimal quantity of 0.4 N NaOH. After taking of the samples for quantitative nitrogen determination, the solution was mixed with an equal volume of veronal buffer (pH 8.6), purified of foreign salts by dialysis for several days against the same buffer, and finally subjected to electrophoresis in an optically recording Tiselius apparatus. In some of the tests the described solution was subjected to electrophoresis and, in others, together with known protein preparations.

On the electrophoregram of the γ-globulin preparation obtained from hot sulfosalicylic acid solution (Fig. 1), it is seen that even after 210 min of electrophoresis, only one sharply defined peak was obtained. This attests to the homogeneity of the preparation. Consequently, in the given case under the influence of heating with sulfosalicylic acid, there does not occur hydrolytic disintegration of the molecule with formation of coarse molecular fragments of the molecule with different electrophoretic mobility.

In order to compare the electrophoretic mobility of the protein preparation obtained with the mobility of the original preparation of γ-globulin which did not undergo heating with sulfosalicylic acid, and also to establish the position of the peak obtained with relationship to the known peak of albumin, there was prepared a mixture of the following three protein preparations which were subjected to electrophoresis: 1) original solution of γ-globulin; 2) a solution of this γ-globulin after treatment with hot sulfosalicylic acid; 3) a solution of pure albumin.

Before mixing of these three preparations, the nitrogen content was determined as indicated. The mixture was so constituted that the concentration of the preparation of interest was greater than the concentration of the other protein preparations. This made it possible to correctly distinguish the peak corresponding to it from other peaks.

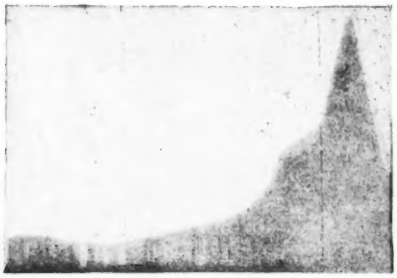

Fig. 3. Electrophoregram of serum albumin after its precipitation from hot sulfosalicylic acid. N concentration – 186 mg%. Descending arm after 205 min from the beginning of electrophoresis; flow: 8 ma, 80 v.

The electrophoregram obtained 188 min after the beginning of current flow (Fig. 2) consists of three peaks. The one furthest to the right in the entire field, peak (A), undoubtedly belongs to comparatively fast-moving albumin. The highest peak (B') characterizes the movement of the γ-globulin taken after treatment with the highest concentration of sulfosalicylic acid. It must be assumed, therefore, that the small peak furthest to the left (B) belongs to the original γ-globulin which did not undergo effects of sulfosalicylic acid treatment.

As can be seen from Fig. 2, the B' peak moves more quickly than peak B. It can therefore be concluded that after precipitation from hot sulfosalicylic acid, the mobility of γ-globulin is somewhat diminished. Therefore, while this cannot be confirmed, it is possible that after solution in hot sulfosalicylic acid, the same molecular structure is preserved as prior to treatment. However, there is no basis to assume a substantial protein decomposition.

Tests similar to those described above were carried out with preparations of serum albumin obtained after cooling of its hot sulfosalicylic acid solution.

In the electrophoregram shown in Fig. 3, appears a peak found characteristic for the albumin position. However, the peak was found to be somewhat flattened in the left-hand portion. This is shown quite clearly by comparison with the sharply confined peak (Fig. 2) which is obtained upon electrophoresis of the original pure albumin preparation. This circumstance attests to the incomplete homogeneity of the preparation obtained, in contradistinction to the complete homogeneity of the original material.

It is of interest to note that such inhomogeneity was not observed in the case of γ-globulin (Fig. 1).

Introduction of Protein Preparations into Sensitized Guinea Pigs

Finally we turn to an account of some preliminary experiments set up so as to clarify the question as to whether anaphylactic properties are preserved by the protein after its treatment with hot sulfosalicylic acid.

Several guinea pigs were sensitized by a single intraperitoneal injection of 0.5 ml human blood serum. From another portion of the same serum protein, preparations were prepared for injection as a permissible preparation. For this purpose 7.5 ml of serum was mixed with 230 ml of distilled water and then with 12.5 ml of 20% sulfosalicylic acid. The liquid together with the precipitate which formed was heated to boiling and filtered hot from the insoluble precipitate. The precipitate of protein sulfosalicylic acid compounds which separated from cold filtrate was dissolved in the smallest possible quantity of 0.4 N alkali and introduced into guinea pigs intracardially on the 14th day after their sensitization by the original serum.

The dosages of the injected solutions, the conditions of the control tests, and the results obtained are given in Table 2.

TABLE 2

Effect of Permissible Injections of Protein Substances on Sensitized Guinea Pigs

Weight of animal	Sensitization	Substance injected on 14th day	Quantity of injected substance in ml	Reaction of animal
		Control		
1045	0.5 ml serum	Original serum	0.5	Typical anaphylactic shock
490	Without sensitization	Serum protein treated with hot sulfosalicylic acid	1	No reaction
		Test		
640	0.5 ml serum	Serum protein treated with hot sulfosalicylic acid	1.2	Strong anaphylactic shock
1080	The same	The same	1.5	Symptoms of anaphylactic shock
840	The same	The same	1.5	Typical anaphylactic shock not observed

As can be seen from Table 2, in one of the three tests a strong anaphylactic shock was obtained, and a weak one in another; in the third test, the reaction was unclear. These results indicate that following precipitation from hot sulfosalicylic acid, the serum protein's anaphylactic properties are preserved almost completely.

DISCUSSION OF RESULTS

From the tests described it can be seen that besides so-called albumoses and proteoses, there exist true proteins, sulfosalicylic acid precipitates of which dissolve readily in hot 1% (approximately 0.04 M) sulfosalicylic acid. This property is far from common in proteins. Corresponding precipitates of γ-globulin and hemoglobin dissolve almost completely in hot sulfosalicylic acid, while precipitates of serum albumin dissolve only insignificantly (6-8%).

We did not investigate the special mechanism of the phenomenon of solution of the precipitate by heating and the reversible precipitation upon cooling, which forms in protein solutions under the influence of sulfosalicylic acid, and therefore we can give only a hypothetical interpretation of this phenomenon.

It is known that precipitates which form upon the addition of sulfosalicylic acid to protein solutions represent protein compounds with the acid. It has been shown that sulfosalicylic acid unites stoichiometrically with the alkaline valences of serum albumin [3]; it was established that in this case the sulfosalicylic acid reacts like a dibasic acid [4]. The authors explain the protein precipitation [4] on the basis of blockading of the cationic groups of the protein by union with the anionic groups of sulfosalicylic acid; this leads to removal of the charges on the protein molecule, hydrophobization of their surfaces and weakening of the linkages of the protein molecule to water.

There have been observations [5] and [6] that upon heating acidified (pH 3) solutions of egg albumin to boiling, the protein does not coagulate and after cooling the solution is shown to be in the original native state. The authors explain this on the basis that decomposition under the influence of heating (denaturation) of the original protein micelles are protected by the acid from irreversible secondary aggregation and that upon cooling assume their prior structure.

Evidently we observed somewhat the same thing, but in relation to combination of the protein with sulfosalicylic acid. It can be assumed that upon heating, these compounds dissociate and the precipitate solubilizes. It is possible that in this way the protein denatures, that is, more of its micelles decompose to some degree; however, the sulfosalicylic acid present impedes the formation of new irreversible molecular links and a precipitate does not appear in the hot protein solution. Upon cooling of the solution there occurs regeneration of the original protein compounds with sulfosalicylic acid.

As to the formation by various proteins of sulfosalicylic acid compounds with different solubilities, evidently this depends upon the chemical structure of the molecule of various types of proteins; it may also be of significance that the isoelectric point of serum albumin lies in the acid range – pH 4.6, while the isoelectric point of γ-globulin and hemoglobin are in a more alkaline region – pH 6-7 [7]. Evidently this contributes to the fact that in acid medium, the sulfosalicylic albumin compounds are more stable than those of globulin.

Under the conditions described, evidently there occurs only a weak hydrolytic effect on heating and boiling, in spite of the presence of sulfosalicylic acid. This conclusion can be made on the basis that after solution in alkali of the protein compound which was heated with sulfosalicylic acid to boiling, the protein preserves a greater homogeneity of its molecule and almost the same mobility under electrophoresis as was inherent in it in the native state; the protein also preserves its antigenic property.

In conclusion we wish to emphasize that the disappearance of the precipitate produced qualitatively with sulfosalicylic acid upon heating cannot serve as a basis for distinguishing albumoses and proteoses from protein. Upon the presence in the solution of some proteins, for example, γ-globulin (in all likelihood also other globulins) and also hemoglobin, these latter as also proteoses are transformed upon heating in solution and consequently the precipitate formed in the cold disappears. Therefore, if this sample were taken for detection of proteose in the case of hematuria in the urine and blood, incorrect results would be obtained. However, as a qualitative reaction as to the completeness of protein precipitation in the cold, it retains its significance.

SUMMARY

A study was carried out on the capacity of sulfosalicylic precipitates of some blood proteins formed in the cold to be dissolved when heated.

The blood proteins were found to behave differently in this respect. The precipitates of γ-globulin and of hemoglobin obtained in the cold in solutions of sulfosalicylic acid (final concentration 1%) dissolve almost completely when the solutions are heated, whereas the precipitate of serum albumin is but slightly dissolved under these conditions (6-10%). Upon cooling of the hot solution, the protein precipitate reappears. Thus it was stated that dissolution of the protein when heated in sulfosalicylic acid is reversible.

Short boiling with sulfosalicylic acid affects but slightly the electrophoretic mobility of the protein particles and their immunobiological properties.

The authors therefore refute the qualitative test as described in the literature for distinguishing albumose and proteose solutions from protein solutions.

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INFLUENCE OF THYROXIN ON CARBOHYDRATE METABOLISM IN THE BRAIN IN CHRONIC EXPERIMENTS

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It is known that thyroxin plays an important role in the mechanism of some neurological disturbances, which occur during hyperthyrosis. An excess of thyroxin disturbs the nerve function parallel with the metabolism of the corresponding tissue, affecting the whole organism. Therefore, it seemed to us of interest to clarify the mechanism of action of this hormone on brain metabolism.

There have been few studies of the functional biochemistry of the central nervous system. Important among them have been the contributions of Soviet scientists. Thus, Palladin and Vladimirov [1] by using methods of radioactive isotopes, established the interrelation between the functional complexity and the intensity of metabolism of substances in various portions of the brain and came to the conclusion that the main source of energy is carbohydrate, even though there is no particularly large reservoir of these substances in the brain. Smirnova and Cetvericov [2] in studying phosphorus metabolism in the brain by using tagged atoms found that the rate of phosphorus metabolism changes in different portions of the brain , and the intensity of exchange of phosphorus compounds between the blood and the brain depends upon the age. Mandel et al. [3] established that, depending upon the age, changes in oxygen and glucose absorption and in formation of lactic acid in the brain were observed. Thus, it appeared that the energy coefficient of combustion of glucose is greater in adults, and anaerobic glycolysis is greater in young animals.

Rabbi and Rassi [4], studying transformation of pyruvic acid in the blood in experimental thyroidism brought about by thyroxin in guinea pigs, found changes directly related to changes in vitamin B.

Among the first scientists who were concerned with the hormone activity on metabolic processes was Academician K. I. Parhon.

In a recent report Parhon et al. [5] showed that incorporation of P^{32} phosphorus in rat brain depended upon their age.

In earlier studies, Milcu et al. [6], studying the effect of thyroxin, caffein and luminal on carbohydrate metabolism, showed the stimulation of such metabolism in the process of glycogen splitting by the first two substances, and its inhibition by luminal. However, these studies [7] showed that the effect of thyroxin introduced in acute tests had a stimulatory effect on carbohydrate metabolism in the direction of glycogen splitting during the stage of anaerobic glycolysis.

The purpose of the present study was to clarify the effect of thyroxin on different stages of carbohydrate metabolism: glycogen splitting, phosphorylation, formation of pyruvic and lactic acid, as well as investigation of the effect of thyroxin upon its protracted introduction.

METHODS

The tests were carried out on 350 rats (adult males) weighing 150 g, who were separated into two groups: the first group received 500 μg thyroxin every 2 days for 20 days.

The second group consisted of normal animals who were killed at the same time as the first group.

Glycogen, amylase, phosphorus compounds, pyruvic and lactic acids were determined in the cerebrum. The procedure for killing was different in different cases. Thus, in determining glycogen, pyruvic and lactic acids, the animals were killed by use of liquid air. Phosphoric esters and amylase activity were determined on rats which were killed by decapitation. The glycogen was determined by the Hinsberg [8] method, amylase by the Engelhardt [9] method, phosphorus compounds by the Meyerhoff [10] method, lactic acid by the Clausen [11] method, and pyruvic acid by the Markees [12] method.

We observed considerable variation in the content of the compounds investigated, depending upon the diet. Thus, for pyruvic acid, the following average values were obtained (for 12 animals):

Animals receiving diet – 2.28 ± 0.13 mg%
Animals fasted 12 hrs – 1.08 ± 0.10 mg%.

From these data it is evident that the quantity of pyruvic acid in animals on the diet comprised 111% of its content in fasting animals. The animals were deprived of food for 12 hrs before being killed.

RESULTS

The results obtained are given in Tables 1, 2, and 3.

The data given in Table 1 permit the following conclusions.

TABLE 1

Glycogen, Amylase, Pyruvic and Lactic Acid Content in Cerebrum of Normal Animals and Hyperthyroid Animals

Substance determined	Normal animals		Test animals	
	determined magnitude	possible variation	determined magnitude	possible variation
Glycogen in mg%	86.41 ± 1.60	76—100	31.25 ± 1.36	24—38
Amylase activity in 100 g tissue	0.605 ± 0.61	0.462—0.680	1.45 ± 0.04	1.20—1.90
Pyruvic acid in mg%	1.65 ± 0.13	1.24—1.98	0.77 ± 0.02	0.60—0.98
Lactic acid in mg%	38.44 ± 1.41	28—54	27.51 ± 1.81	13—31
Ratio lactic acid/pyruvic acid	23.29	16—34	37.02	21—68

TABLE 2

Changes in Content of Phosphorus Compounds in the Brain Under the Influence of Thyroxin

Substance	Normal animal* (control)	Test animal*
Inorganic phosphorus	34.66 ± 0.03	49.75 ± 0.05
Hexosephosphate ester	15.34 ± 0.05	24.66 ± 0.03
Diphosphoglyceric acid	10.00 ± 0.01	24.00 ± 0.03
Total acid soluble phosphorus	62.00 ± 0.02	103 ± 0.04

* Number of tests for each group of animals – 6.

The glycogen content in the cerebrum of normal animals and in animals which were given thyroxin confirmed results obtained by us previously [6, 7]. In the given cases there was also observed a decrease in the glycogen content in the brain under the influence of thyroxin (63% by comparison with normal values).

The actual data obtained by us for glycogen in the large hemispheres of the brain of normal rats (86.41 ± 1.60 mg%) are close to the data obtained by Palladin [13] for whole rabbit brain (114 mg%).

The amylase activity in normal rat brain under the influence of thyroxin increases from 0.605 ± 0.01 to 1.45 ± 0.04, that is, by 139%. The increase in amylase activity takes place at the same time as there is a decrease in the glycogen content.

The pyruvic acid content in the large hemispheres of the brain in normal rats comprises 1.65 ± 1.13 mg%. Under the influence of thyroxin there is observed a decrease in pyruvic acid content in the large hemispheres of the brain to 0.77 ± 0.02 mg%, or by 53% by comparison with the average normal values.

Thyroxin effects a decrease in the lactic acid content in rat brain from 38.44 ± 1.42 (in normal rats) to 27.51± 1.81 mg%, that is, by 45%. The values obtained for lactic acid in rat cerebrum are of the same order as values obtained by Palladin [13] for whole rabbit brain.

The ratio of lactic acid/pyruvic acid (which characterizes direct anaerobic glycolysis) constitutes 23.29 in normal animals, and under the influence of thyroxin increases to 37.02. Thus, under the influence of thyroxin, an increase in anaerobic glycolysis by 45% occurs.

Data as to the change in content of phosphorus compounds in the brain under the influence of thyroxin are shown in Table 2, indicating the following.

The phosphorus content of hexosephosphate esters in the brain which in normal animals constitutes 15.34± ± 0.5 mg%, increases to 24.66 ± 0.03 mg% in the test animals. The increase corresponds to 73%.

The diphosphoglyceric acid content in the cerebrum increases from 10.00 ± 0.01 mg% (control) to 24.00 ± ± 0.03 mg% (test), under the influence of thyroxin, which corresponds to 140%; the total acid soluble phosphorus from 62.00± 0.02 mg% to 103± 0.04 mg%, which corresponds to an increase of 65%; inorganic phosphorus from 34.66 ± 0.03 mg% to 49.75± 0.05 mg%, that is, by 44%.

TABLE 3

Effect of Thyroxin on Various Stages of Anaerobic Glycolysis in Rat Brain

Substance determined	Normal animal	Test animal
	Glycogen splitting	
Glycogen in mg%	86.30±1.60	31.25±1.36
Amylase activity in 100 g tissue	0.605±0.01	1.45±0.04
	Phosphorylation	
Hexosephosphate ester in mg%	15.34	24.66
Diphosphoglyceric acid in mg%	10	24
Pyruvic acid in mg%	1.65±0.13	0.76±0.02
Lactic acid in mg%	38.70±1.42	20.93±1.81

Table 3 gives results of the effect of thyroxin on various stages of anaerobic glycolysis. Results similar to the ones shown were obtained for normal rat brain.

We observed that, under the influence of thyroxin, there occurs a stimulation of various stages of anaerobic glycolysis; an increased glycogen splitting along with an increase in amylase activity, and stimulation of phosphorylation.

DISCUSSION OF RESULTS

The data obtained confirm individual results obtained by us previously. In addition, we obtained new data concerning the effect of thyroxin on other stages of carbohydrate metabolism in the brain: the process of phosphorylation (with the assistance of investigation of intermediate phosphorus compounds) and the formation of lactic and pyruvic acid at the stage of anaerobic glycolysis.

Thyroxin introduced in chronic experiments influences carbohydrate metabolism at various characteristics stages of anaerobic glycolysis.

The effect of thyroxin is manifested at various stages of metabolism.

1. There occurs a stimulation of glycogen splitting, which is manifested in a decrease in glycogen content in the brain together with an increase in amylase activity. This result corresponds to the data obtained by Rashba [14], which showed that the brain amylase splits 50-70% of polysaccharides. He established the presence of maltose in a brain suspension, the hydrolysis of which depends upon the action of maltase.

2. In the process of phosphorylation, the effect of thyroxin is manifested in an increase in the phosphorus content in intermediate compounds (hexose esters, diphosphoglyceric acid) and an increase in phosphoric acid content.

The lability of phosphorus compounds has been indicated in the literature. Thus Bieth et al. [3] found variations in the content of acid soluble phosphorus compounds in rat brain during growth.

3. Finally, the effect of thyroxin is manifested in a stimulation of anaerobic glycolysis, which is expressed by an increase in the ratio of lactic acid/pyruvic acid by 59%, by comparison with the value obtained in normal animals.

It is known that thyroxin stimulates carbohydrate metabolism in the brain in the stage of anaerobic glycolysis At the same time it has been observed that this metabolism is characterized by a greater lability and intensity.

In studying the effect of various stimulatory factors on carbohydrate metabolism, Khaikina et al. [15] noted that under the influence of pervitin or cardiazol , introduced in acute experiments, there is an increase in the glycogen content as a result of the synthetic activity of phosphorylase, but upon continued introduction of these substances there occurs,on the contrary, a decrease in the glycogen content in the brain.

By stimulation of the central nervous system by the use of camphor, Vladimirov [16] brought about an increase in products of glycolysis. Parallel with an increase in the glycogen content of the brain, there occurs an increase in amylase activity and in inorganic phosphorus.

Upon stimulation through the use of pervitin, Palladin [13] detected a decrease in the lactic acid content in the brain by 15%, and following prolonged stimulation a decrease in the content of polysaccharides with a simultaneous increase in the quantity of enzymes upon which their cleavage depends. On the other hand, Etting [17] showed that substances which bring about hypnosis have no influence on the mechanism of phosphorylation, isomerization, and also on the cleavage of fructose diphosphate in the brain, and they inhibit only dehydrogenation of lactic acid which leads to aerobic accumulation of this compound.

The significant decrease in lactic acid content under the influence of thyroxin (by 29%) in our tests may be explained by the increase in dehydrogenation of this compound in the brain.

In studying the effect of narcosis, Ivanenko [18] noted a decrease in the content of organic phosphorus in rat brain. In his tests conducted with thyroxin, we did not observe a difference in the direction of change obtained in chronic and acute experiments; new data indicate only an increase in stimulation of carbohydrate metabolism under the chronic influence of thyroxin.

Vaccari and Maloguti [19], studying the effect of desoxycortisone, cortisone and pregnandiol on brain carbohydrate, noted that cortisone acetate and pregnandiol acetate, which do not bring about narcosis, have no influence on carbohydrate metabolism , while the acetate and glucoside of desoxycortisone, which bring about narcosis, increase the total carbohydrate content of the brain.

Thus the literature shows that stimulation of carbohydrate metabolism in the brain is observed upon stimulation of the nervous system and inhibition of this metabolism by the effects of substances which bring about narcosis.

These data, compared with the results we obtained, indicate the benefit of stimulation of carbohydrate metabolism under the influence of thyroxin introduced under the described conditions.

The processes which precede the formation of lactic acid from pyruvic acid are known. However, the region of investigation of the subsequent processes are characterized by divergent opinions, since the corresponding enzymes have been little studied.

The decreased content of lactic and pyruvic acid in our tests after introduction of thyroxin in chronic experiments indicates stimulation of carbohydrate metabolism in the stage of anaerobic glycolysis, and the acceleration of metabolism of pyruvic acid in the aerobic stage. According to Elmer Stoltz [20], the main metabolic path

for pyruvic acid metabolism(as its oxidation, decarboxylation and condensation)evidently requires the presence of one or another enzyme. Upon the partial loss of the latter, as in B_1 avitaminosis, there accumulates a larger quantity of pyruvic acid in the blood and tissues. From the literature, the importance of pyruvic acid metabolism for the synthesis of acetylcholine in the brain is evident.

SUMMARY

Thyroxin introduced in chronic experiments influences various processes of carbohydrate metabolism in the brain.

The effect of thyroxin is manifested by stimulation of carbohydrate metabolism in the stage of anaerobic glycolysis. Thyroxin brings about a strengthening of the process of glycogen splitting, parallel with an increase in amylase activity, stimulates the process of phosphorylation and strengthens glycolysis.

Thyroxin brings about an acceleration of pyruvic acid metabolism.

These phenomena show that carbohydrate metabolism in the brain is characterized by special lability and intensity.

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THE AMYLOLYTIC ACTIVITY OF THE SKIN*

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In the course of our previous investigations [1], it was shown that guinea pig skin extracts and rabbit skin extracts are capable of splitting both starch and glycogen. It was established that the amylase contained in the skin is activated by chlorides and globulins, and is suppressed by mercuric chloride and by resorcinol.

The aim of the present work was to pursue further investigation of the splitting of carbohydrates in the skin, under the action of amylase, and to obtain evidence that this cleavage could take place without the participation of phosphorylase.

METHODS

The investigations were conducted using fresh and dialyzed skin extracts from adult rabbits and guinea pigs. Skin freed from its hairy integument and subcutaneous fatty tissue was passed through a meat grinder chilled in a refrigerator; after standing for an hour at low temperatures, the minced meat was added to an equal volume of chilled distilled water. Extraction was carried out for 30 min with frequent agitation, and the liquid was then expressed through gauze. The extract was centrifuged and dialyzed again for 2 days in distilled water at 5-8°.

The investigations were carried out without buffer as a rule, since the pH for the dialyzed extracts was always adjusted to within the optimum range for animal amylase (6.8-7.0).

Amylase activity was determined according to the amount of reducing substances stored, whereby we took into account both dextrination and saccharification.

Reducing substances were determined according to Hagedorn-Jensen, phosphorus according to Fiske-Subbarow. The formation of dextrins was estimated on the basis of an iodine sample.

RESULTS

1. Determination of Phosphorus

In order to establish conclusively that the cleavage of starch or glycogen by rabbit and guinea pig skin extracts can proceed due to the action of amylase alone without enzymes of the phosphorylating system taking part, we conducted experiments on the splitting of starch and glycogen with simultaneous determination of reducing substances and inorganic phosphorus.

The data obtained are presented in Table 1.

From the data in Table 1, it follows that the increase in reducing substances present is not accompanied by any drop in inorganic phosphorus, which attests to the absence of phosphorylase under the conditions of our experiments.

Since these specially performed determinations of the amount of inorganic phosphorus present in the dialyzed extracts which we used demonstrated that inorganic P was practically negligible, phosphate buffer was added to the experimental mixture in the experimental part. In some of the experiments, sodium fluoride was added as an inhibiting agent to retard the activity of phosphatases.

* G. M. Antonova took part in the experimental part of the work.

TABLE 1

Cleavage of Starch by Rabbit Skin Extracts

Composition of experimental mixture: 20 ml extract + 4 ml 1% starch + 108 mg sodium chloride (Experiment A) or 210 mg sodium fluoride (Experiment B). Incubation at 37°, pH 6.8-7.0 .

Time of incubation, min	Color of solution	Reducing substances in mg/ml	Inorganic P, mg/ml	Time of incubation, min	Color of solution	Reducing substances in mg/ml	Inorganic P, mg/ml
Experiment A				Experiment B			
0	Dark blue	0.25	0.027	0	Dark blue	0.19	0.032
30	Lilac	0.33	—	30	Lilac	0.41	—
60	Red	0.61	0.023	60	Violet	0.50	0.025
120	Colorless	0.63	0.023	120	Red	0.58	0.025
240	Colorless	0.80	0.027	240	Colorless	0.78	0.032

TABLE 2

Cleavage of Starch and Glycogen by Rabbit Skin Extracts Under Various Conditions

Composition of experimental mixture	Experiment									
	1		2		3		4		5	
Extract, in ml	5		5		15		10		6	
1% Starch in ml	1		—		3		4		—	
2% Glycogen in ml	—		1		—		—		5	
1% Sodium chloride in ml	—		—		1		—		—	
Sodium fluoride, ml	—		—		—		10		38	
Phosphate buffer in ml	0.6		0.6		0.37		—		3	
Water, ml	0.4		0.4		—		—		2	
Time of incubation in min	reduction	phosphate	reduction	phosphate	reduction	phosphate	reduction	phosphate	reduction	phosphate
0	0.47	0.167	0.40	0.144	0.14	0.059	0.39	0.055	—	0.255
30	0.84	0.135	0.98	0.125	0.22	—	1.33	0.061	—	—
60	0.94	0.213	0.29	0.178	0.33	0.052	1.56	0.054	—	0.054
120	1.03	0.135	1.48	0.145	0.38	0.046	1.71	0.073	—	—
240	0.96	0.123	1.42	0.145	0.55	0.046	1.72	0.058	—	0.232

Note: Values for reducing substances and inorganic phosphorus in mg/ml.

Results were obtained (Table 2) analogous to those presented in Table 1: a considerable increase in reduction and the absence of any regularly occurring drop in inorganic phosphorus were also observed.

2. Effect of Phlorizin on Starch Cleavage

The following evidence of the presence of amylase in the skin was provided by experiments using phlorizin which, as is known, stops the phosphorolysis of starch and glycogen.

The results obtained are to be found in Table 3.

Table 3 makes it clear that, in the presence of phlorizin, hydrolysis of starch takes place; reduction increases in all three of the experiments. No loss in inorganic phosphorus was detected, attesting to the complete suppression of phosphorolysis.

TABLE 3

Effect of Phlorizin on Hydrolysis of Starch by Guinea Pig Skin Extracts.
Composition of experimental mixture: 5 ml extract + 3 ml 1% starch + 0.6 ml phosphate buffer + 1.4 ml water + phlorizin; 25 mg (Experiment A), 50 mg (Experiment B), or 60 mg (Experiment C). Incubation, 37°; pH 6.8-7.0.

Time of incubation, min	Reducing substances, mg/ml	Inorganic P, mg/ml	Time of incubation, min	Reducing substances, mg/ml	Inorganic P, mg/ml	Time of incubation, min	Reducing substances, mg/ml	Inorganic P, mg/ml
Experiment A			Experiment B			Experiment C		
0	2.01	—	0	2.89	—	0	2.89	0.268
30	—	—	30	—	—	30	—	—
60	2.47	—	60	3.44	—	60	3.44	0.325
120	2.78	—	120	2.72	—	120	3.72	0.314
240	2.66	—	240	2.93	—	240	3.92	0.300

In that manner, experiments using phlorizin also confirmed the presence of amylase in the skin of guinea pigs.

3. Activity of Skin Extracts in the Presence of Medinal Buffer

The considerable increase in reducing substances during incubation in the experiments with skin extracts might be the result of both amylase activity and the subsequent action of two enzymes, phosphorylase and phosphatase. To clear up that question, we carried out experiments with guinea pig skin extracts in the presence of medinal buffer, i. e., under conditions of almost complete exclusion of phosphate from the experimental sample.

TABLE 4

Amylase Activity of Guinea Pig Skin Extracts

Time of incubation, hrs			
0	1	2	4
reducing substances, mg%			
47	81	112	—
50	93	108	129
29	79	87	120

The conditions for carrying out the experiment were the following. Composition of the experimental mixture: 10 ml extract + 2 ml 1% starch + 0.5 ml 10% NaCl + 2 ml medinal buffer, pH 7.2. Incubation at 37°.

Under the experimental setup described, the possibility of phosphorolysis was, for all practical purposes, excluded.

From the results cited in Table 4, it follows that, despite the exclusion of the phosphorolytic process, the increase in reducing substances took place.

This fact justifies the conclusion that hydrolysis of carbohydrates is to be attributed precisely to the amylolytic activity of the skin extracts.

4. Effect of Mercuric Chloride on Skin Amylase Activity

Taking into account the fact that mercuric chloride exerts an inhibitory effect on amylase activity without affecting the activity of phosphorylase or of the phosphatases, we conducted a series of experiments in which mercuric chloride was added to the incubation mixture.

From the results obtained, given in Table 5, it follows that mercuric chloride inactivates skin amylase. The increase in reduction was not observed, while the absence of any loss in inorganic phosphorus suggests that under our experimental conditions, phosphorylase was without effect.

5. Effect of Presence of Blood Residue on Skin Amylase Activity

Since blood exhibits a rather active amylolytic capacity, it was naturally suggested that amylase activity in skin extracts was due to the small residue of blood which might possibly have been retained in the skin under

investigation. Along this line, a portion of the skin, in one series of experiments, was carefully washed free of blood residue, using physiological solution, and a second portion was partially washed, a third portion of skin not being washed at all. An extract was prepared from each portion, the extract was mixed with an equal volume of medinal buffer (pH 7.2) containing 10 mg glycogen.

TABLE 5

Effect of Mercuric Chloride on Amylolytic Activity of Rabbit and Guinea Pig Skin Extracts. Added to all samples: 1 ml 3% mercuric chloride and 0.6 ml phosphate buffer, pH 6.8. Incubation at 37°. Rabbit skin extract was used in Experiments 1-5; guinea pig skin extracts in Experiments 6-7. Reduction and phosphate in mg/ml.

Composition of exptl. mixture	Experiment													
	1		2		3		4		5		6		7	
Extract in ml	5		5		5		5		4		4		5	
1% starch in ml	2		2		2		2		1		1		2	
10% sodium chloride in ml	0.5		0,5		—		—		—		—		0.5	
Sodium fluoride in mg	—		50		—		100		—		50		—	
Time of incubation, hrs	reduction	phosphate	reduction	phosphate	reduction	phosphate	reduction	phosphate	reduction	phosphate	reduction	phosphate	reduction	phosphate
0	0.37	0.248	0.35	0.213	0.32	0.310	0.25	0.082	0.45	0.263	0.33	0.176	0.36	0.270
1/2	0.39	0.324	0.41	0.246	0.37	0.348	0.40	0.097	—	—	—	—	0.40	—
1	0.41	0.413	0.48	0.257	0.33	0.282	0.37	0.148	0.41	0.204	0.34	0.121	0.68	0.350
2	0.43	0.300	0.42	0.192	0.43	0.320	0.40	0.125	0.40	0.252	0.34	0.161	0.40	0.269
4	0.44	0.333	0.43	0.230	0.41	0.364	0.44	0.174	0.40	0.260	0.41	0.151	0.50	0.280

TABLE 6

Effect of Presence of Blood Residue on Guinea Pig Skin Amylase Activity.
Composition of experimental mixture: 1 ml fresh extract + 1 ml medinal buffer + 10 mg glycogen. Incubation at 37°.

Time of incubation in hrs	Reducing substances, in mg%				
	22*	23	24	25	26
Unwashed skin					
0	22	17	51	73	11
1	156	102	89	166	137
2	—	—	122	196	169
Increase	134	85	71	123	158
Partially washed skin					
0	21	10	48	57	12
1	153	90	110	166	137
2	—	—	143	185	182
Increase	132	80	95	128	170
Well washed skin					
0	12	10	38	49	13
1	126	90	87	155	160
2	—	—	133	178	201
Increase	114	80	95	129	188

* Number of pigs.

The data obtained, tabulated in Table 6, show that no essential difference was observed in the increase of reducing substances in experiments with unwashed skin when compared with experiments where the skin was well washed, and in particular when compared with partially washed skin. When it is taken into account that, in all the investigations of amylolytic activity on the part of the skin, the skin preparations were first always washed with physiological solution, the conclusion then follows that the amylase activity of skin tissue is not to be assigned to any residue of blood contained in the skin samples under study.

Accordingly, the data cited in the present discussion and those published earlier [1-6] demonstrate that the main pathway for the breakdown of carbohydrates in the skin should be understood to be the amylolytic pathway, and not the phosphorolytic route.

SUMMARY

It was shown in experiments with fresh and dialyzed aqueous extracts of rabbit and guinea pig skin, that the chief pathway of carbohydrate breakdown in the skin is the amylolytic, rather than the phosphorolytic pathway.

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A STUDY OF PEROXIDASE AND CATALASE ACTIVITY OF PENICILLIUM CHRYSOGENUM Q-176 WITH REFERENCE TO THE ACCUMULATION OF PENICILLIN IN THE CULTURE MEDIUM

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Our knowledge of the essence of the enzymatic processes taking place in mold fungi is based for the most part on the result of studies of analogous transformations in bacteria and yeast. An insufficient fund of information relating to the formation of penicillin and its links with the enzymatic processes taking place in Penicillium cells led us to undertake research on the biochemical activity of the mold cells.

We felt it interesting to compare the dynamics of penicillin production in Penicillium chrysogenum Q-176 with the change in peroxidase and catalase activity in the cells and in the culture medium. The present discussion is devoted to the elucidation of the results of work conducted along that line.

METHODS

The mold enzyme studies were carried out with growing of the mold in both surface and submerged phases. Surface cultivation was carried out in standard vessels over a 15-day period. In cultivation in depth, the mold was grown in special cylindrical containers; air was passed through the culture fluid in amounts of 2 liters/min, measured with a rheometer. The cultivation was extended over 9 days. Under these conditions of production, the overall duration of mold cultivation extended to 5 days, including preliminary cultivation.

The composition of the nutrient media used was the following: lactose – 20 g, dextrose – 5 g, ammonium sulfate – 5 g, calcium hydrogen phosphate – 2 g, magnesium sulfate – 0.5 g, ferrous sulfate – 0.05 g, zinc sulfate – 0.04 g, manganese sulfate – 0.02 g, copper sulfate – 0.0005 g, acetic acid – 4 ml, distilled water – 1 liter.

Studies of the mold mycelium and of the culture fluid were carried out over different periods of mold growing; the following indices were investigated: 1) peroxidase and catalase activity, 2) penicillin content in the culture fluid, 3) variation in the dry weight of the mold), 4) variation in the pH values of the culture medium. Samples were taken daily for analysis. The average sample consisted of the contents of three containers. Under the conditions of production observed, samples were taken from the mass in a ten-ton tank.

Determination of peroxidase and catalase activity was carried out on fresh material immediately after the sample was taken. Mold film, or submerged mycelium, were washed well with distilled water and drawn off by suction on a Büchner funnel. The sample of dry fungal mass was crushed in a porcelain mortar and then left to stand in 100 ml distilled water for 2 hrs at room temperature. 10 ml of the suspension so prepared had 100 ml of distilled water added to it, and samples were taken from the solution for determination of enzyme activity. Culture fluid for the determination of enzyme activity was added in proportions of 1:10. Peroxidase activity was determination according to the method of Bakh and Zbarski, catalase activity, according to the method of Bakh and Oparin. Penicillin activity in the culture fluid was determined by the method of batch dilution in the course of its action on Staphylococcus aureus, and the activity was expressed in terms of units per 1 ml. pH was determined by using a potentiometer.

RESULTS

1. Features of Peroxidase and Catalase Reaction in Penicillium Chrysogenum Q-176

It is well known from the literature that a number of enzymes are firmly bound to the cell protoplasm [1, 2], and that, in order to free them, it is necessary to use either prolonged autolysis [3] or freezing with subsequent rapid thawing [4], or else treatment with a buffer salt solution at a fixed pH value [5].

In studying the enzymes of Penicillium chrysogenum Q-176, we succeeded in establishing that the most effective of these procedures, for the purpose of arriving at the peroxidase and catalase activity, was the use of phosphate buffer solutions having optimal pH value.

It was revealed that peroxidase in aqueous suspension (pH 6.9-7.1), prepared from a fungal mycelium, exhibited a very low activity, while catalase from the same suspension showed a high activity.

The experimental findings showed that, in surface cultivation of the mold, the maximum enzymatic activity was detected on the 9th day of growing. Peroxidase activity of the aqueous suspension of mold was found to be only insignificant, being completely absent within 3 to 15 days. In the culture fluid, maximum peroxidase activity was also determined on the 9th day, with the lowest amount on the 15th day and complete absence during the first days of growth.

Catalase from the same mycelial sample displayed considerable activity over the entire course of the experiment, and the quantity of it secreted in the culture fluid increased during the process of mold growth. The pH value of the culture medium varied during the period of mold growth from 6.17 to a high of 8.00. These pH values of the medium corresponded to optimal activity for both enzymes.

TABLE 1

Effect of Different pH on Peroxidase Activity of Aqueous Suspension from Penicillium chrysogenum Q-176 Mycelium Cultivated in Depth. Peroxidase activity expressed in ml 0.1 N $KMnO_4$.

pH with phosphate buffer absent	pH with phosphate buffer added							
7.0	6.47	6.98	7.38	7.73	8.04	8.31	8.67	9.18
10.14	203.9	310.2	458.4	478.6	520.8	529.3	554.5	571.3

To establish the actual peroxidase and catalase activity in the mycelium and culture fluid, phosphate buffer solution was added to the reaction mixture in proportions of $^1/_{10}$ volume of experimental mixture. Peroxidase activity in the mycelium then rose by a factor of 10 or more; its activity in the culture field showed no change in the presence of the buffer solution. Catalase activity also failed to show a change in response to the introduction of buffer solution, both in the suspension and in the culture fluid. The optimal pH value for peroxidase activity in the mold mycelium was established on addition of the phosphate buffer (Table 1).

From the data cited (Table 1), it follows that the detection of the actual peroxidase activity in the mold mycelium under study was facilitated by the introduction of phosphate buffer solutions at any of the given pH values. An especially high activity of this enzyme was recorded on addition of buffer shifting the pH value to the 8.0-9.2 range.

2. Changes in Penicillium chrysogenum Q- 176 Peroxidase and Catalase Activity During Accumulation of Penicillin in the Culture Fluid

Establishing the interconnection between the physiological state of the mold, the features of its enzyme activity, and penicillin formation was an interesting and vital question relating to penicillin biosynthesis. Observation of peroxidase and catalase activity of the mold in the course of its protracted cultivation in the accurately known and easily regulated synthetic medium used provided a possibility of studying the regularity and trend of the action of these enzymes during the period of accumulation of mycelial mass and subsequent autolysis.

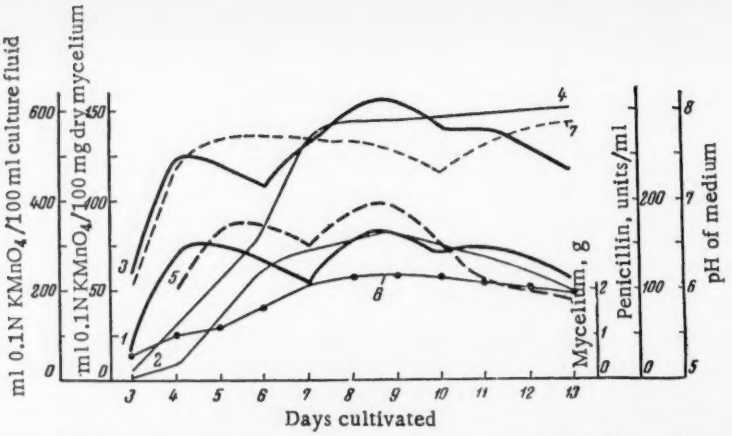

Fig. 1. Activity of peroxidase, catalase, penicillin, dry weight of mycelium and pH of culture fluid of Penicillium chrysogenum Q-176 cultivated at surface: 1) mycelium peroxidase; 2) culture fluid peroxidase; 3) mycelium catalase; 4) culture fluid catalase; 5) penicillin in culture fluid; 6) dry weight of mycelium; 7) pH of culture medium.

Figure 1 illustrates the results of studies on the variation of peroxidase and catalase activity, as well as on other indices concerned with the process of accumulation of penicillin in the culture fluid in surface cultivation.

Biochemical studies of the mold were begun only on the third day after inoculation (Fig. 1), when the very thin film extended over the surface of the liquid medium.

According to the extent of development of the mold, a gradual accumulation of the mycelial mass took place, reaching its maximum value at the 8-9th day after inoculation. The weight of the mycelium gradually dropped off in line with the autolysis of the mold which was setting in. Peroxidase and catalase activity in the mycelium, contained in 100 ml of culture medium, was negligible by the third day. On the fourth and successive days, the activity of these enzymes was on the increase and reached its maximum by the 8-9th day. After attaining maximum value, the activity of the enzymes decreased.

The activity of both enzymes in the mycelium increased almost simultaneously, and reached the maximum and declined at the same time, which evidently points to a coupling of their action. The change in peroxidase activity in the culture fluid repeated almost the identical trend, as in the mycelium: at 8-9 days of mold cultivation, the activity reached its maximum and then fell off.

TABLE 2

Peroxidase and Catalase Activity, Accumulation of Penicillin and Change in pH of Culture Fluid in Submerged Cultivation of Penicillium chrysogenum Q-176, Laboratory experiment.

Days of cultivation	Weight of dry mycelium, in g/300 ml of culture fluid	Penicillin, in units/1 ml	Activity, in ml 0.1 N $KMnO_4$						pH of culture fluid
			peroxidase			catalase			
			in 100 mg of dry mycelium	in 100 ml of culture fluid	per dry mycelium, in 100 ml culture fluid	in 100 mg of dry mycelium	in 100 ml of culture fluid	per dry mycelium, in 100 ml culture fluid	
3	1.00	16	49.0	213	163	32.7	36	108	6.50
4	1.92	80	315.0	321	2016	190.0	261	1216	7.00
5	2.48	160	451.5	612	3733	241.2	433	1994	7.30
6	2.30	240	628.0	1277	4816	289.0	986	2216	7.70
7	2.05	160	446.0	1635	3046	250.0	1003	1707	7.50
8	1.90	120	418.5	2168	2649	229.0	1295	1449	7 80
9	1.71	80	412.0	2501	2348	211.0	1350	1202	7.87

Catalase activity in the culture fluid differed from peroxidase activity in that after reaching its high point at the 8th and 9th days, it did not decline, but remained at a high level.

Parallel to the activity curves for the enzymes in the mycelium is the slope of the curve showing penicillin accumulation in the culture fluid, reaching the maximum value at 8-9 days. Accumulation of penicillin decreased together with the drop in enzyme activity.

The pH of the culture medium, equal to 6.0 when the mold was inoculated into the medium varied during the process of growth and development of the mold and by the 15th day of cultivation reached the value 8.0, with some fluctuations accounted for.

In submerged cultivation, the development of the fungal biomass and the metabolic processes were more intense than in the case of surface cultivation. In the case of the submerged culture, we observed an even more sharply expressed parallelism between peroxidase and catalase activity in the mycelium and penicillin activity in the culture fluid.

The results of the peroxidase and catalase activity studies in the process of penicillin accumulation in the culture fluid on cultivation of the mold in depth are tabulated in Table 2.

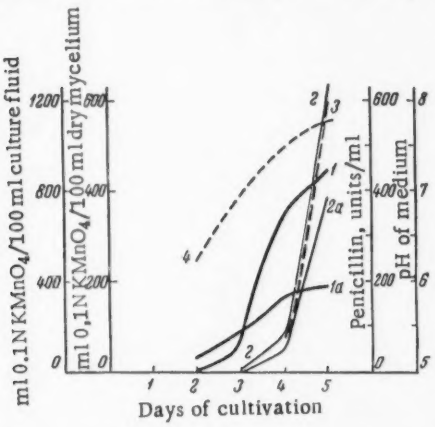

Fig. 2. Activity of enzymes and of penicillin of Penicillium chrysogenum Q-176 in depth cultivation under conditions of production: 1, 1a) peroxidase and catalase in the mycelium; 2, 2a) peroxidase and catalase activity in the culture fluid; 3) penicillin activity; 4) pH of medium.

Despite the difference manifest under conditions of surface and depth cultivation of the mold, the activity of its enzymes, as well as the accumulation of penicillin in the culture fluid, retained similar features of regularity, for the most part, in both of these techniques of cultivation.

The experiments under discussion (Table 2) demonstrated that peroxidase and catalase activity in the mycelium increased to the maximum on the 6th day (by factors of 13 and 9, respectively, for peroxidase and catalase, compared to the 3rd day). The activity of the enzymes in the culture fluid gradually increased and rose considerably (ten times the 3rd day) on the 9th day of cultivation. Penicillin activity in the culture fluid varied with respect to rise and fall of enzyme activity in the mycelium. The mycelial mass of the mold rose to its maximum on the 5th day, after which its weight showed a gradual decline. The pH value of the culture fluid varied with some fluctuations from 6.5 to 7.87.

The indices so obtained gave us a chance to establish the regular features of Penicillium chrysogenum Q-176 peroxidase and catalase action, and also to get a picture of the production of penicillin during the period of its normal development under the conditions of our experiments.

In subsequent investigations, it was required to elucidate whether or not the regularities of peroxidase and catalase action, as revealed in the laboratory experiments, on cultivation of the mold under the conditions of production described, were retained.

The experimental findings cited below (Fig. 2) proved to be typical of the submerged culture of Penicillium chrysogenum Q-176, grown under conditions of production.

The activity of the enzymes in Fig. 2 is related to the amount of the mycelium contained in 100 ml of the culture medium, as a consequence of the fact that the ratio of the mycelium to the culture fluid underwent some variation during the course of the growth process of the mold.

All processes accompanying the growth of the mold, under these conditions, proceeded at a notably more intense rate (the period of fermentation extended for 72 hours) than in the depth culture in the laboratory experiments.

The activity of the mold enzymes, and of peroxidase in particular, was low in the inoculum, rising sharply in 48 hrs, and reaching a maximum in 72 hrs. The same sharp rise in peroxidase and catalase activity was noted in the culture fluid as well, but some days later. The curve of penicillin activity in the culture fluid showed a slope almost parallel to that of the peroxidase activity curves in the mycelium and in the culture fluid, and approximately parallel to the activity curve for catalase. The pH value fluctuated during the growth process of the fungus from 6.2 to 7.8. The accumulation of the biomass (not indicated in Fig. 2) attained its maximum value toward 48 hrs of fermentation.

Thus, in the course of investigations of Penicillium chrysogenum Q-176, grown under production conditions, the same regularity of increase in peroxidase and catalase activity was duplicated, as was the accumulation of penicillin to the high point value observed also in the laboratory experiments.

When the mold was cultured under submerged conditions just as in surface cultivation, we had to deal with the fact of impairment of the optimal conditions of the set of circumstances necessary for the normal growth of the mold and for its production of penicillin. This was usually accompanied also by the impairment of the regularity observed in changes of peroxidase and catalase activity in the mycelium. The formation of penicillin was thereupon hampered and the maximal yield was shifted to a later period or depressed appreciably.

Data from the laboratory experiments are given in Fig. 3.

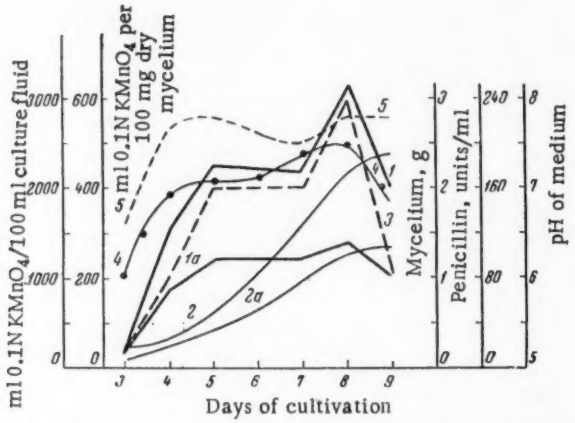

Fig. 3. Activity of peroxidase, catalase and penicillin, dry weight of mycelium and pH values in submerged culture of Penicillium chrysogenum Q-176 with processes retarded: 1, 1a) peroxidase and catalase of mycelium; 2, 2a) peroxidase and catalase in culture medium; 3) penicillin; 4) dry weight of mycelium; 5) pH of culture medium.

The experimental results (Fig. 3) show that peroxidase and catalase activity in the mold increased to the maximum only on the 8th day of cultivation, which was a deviation from the norm, compared to the data from the earlier experiments. The curve of penicillin accumulation ran parallel to the activity curves of the enzymes in the mycelium, with a slowing up on the 6-7th day. The mass of the fungal mycelium increased gradually up to the 5th day, with a retardation observed on the 6th day, and reached its maximum value toward the 8th day. The pH of the culture medium shifted to the alkaline side on the 4th day, remaining on one level up to the 6th day, and then rose to 8.0.

A contribution to the impairment of the processes described was given by the insufficient aeration ensuing from a temporary blockage of air intake on the 4th day of cultivation of the mold.

Similar results were obtained by Japanese authors [6, 7] studying catalase activity in Penicillium chrysogenum Q-176. According to their findings, catalase activity at first increases rapidly, reaching its high point in the middle phase, and then begins to drop off slowly. In cases where aeration was cut off and stirring was begun 42 hrs after fermentation began, catalase activity in the culture fluid showed a steady decline. Penicillin formation came to a halt in the process. The view of the authors is that catalase plays some definite role in penicillin formation.

In laboratory tests on submerged cultures of Penicillium chrysogenum Q-176 we discovered that the impairment, in the culture medium, of even one of the factors contributing to the normal development of the mold (aeration or optimum pH) led to a marked change in enzyme activity. Penicillin formation was retarded and the yield was diminished.

Table 3 provides a comparison of the data of two experiments, in one of which fermentation of the mold proceeded normally and formation of considerable quantities of penicillin was observed; in the other experiment, a sharp drop in the pH value of the medium was observed, along with a low penicillin yield.

TABLE 3

Peroxidase and Catalase Activity in Submerged Culture of Penicillium chrysogenum Q-176 and Accumulation of Penicillin Under Normal and Disturbed Conditions of Fermentation. Experiment carried out under production conditions. Activity of enzymes expressed in ml 0.1 N $KMnO_4$.

Conditions of cultivation	Days of cultivation	Peroxidase		Catalase		Penicillin in units per 1 ml	pH of culture medium
		in 100 mg dry mycelium	in 100 ml culture fluid	in 100 mg dry mycelium	in 100 ml culture fluid		
Normal fermentation conditions							
Preliminary cultivation in bottles	1	—	—	—	—	—	—
The same	2	4.2	0.0	10.2	0.0	—	6.2
Cultivation in large tanks	3	45.0	0.0	75.0	0.0	—	6.8
The same	4	351.4	98.0	175.7	152.5	80	7.5
The same	5	450.0	1235.0	183.5	778.0	640	7.8
Disturbed fermentation conditions							
Preliminary cultivation in bottles	1	—	—	—	—	—	—
The same	2	33.7	0.0	52.5	15.3	—	6.2
Cultivation in large tanks	3	167.1	0.0	117.3	61.0	—	6.2
The same	4	300.0	46.0	144.2	152.5	80	4.38
The same	5	435.5	679.0	173.6	336.0	120	4.38

In comparing the data of the two experiments (Table 3), it becomes clear that impairment of the optimal conditions for culturing Penicillium chrysogenum Q-176 leads to a sharp drop in penicillin yield. When fermentation was impaired, an enhancement of peroxidase and catalase activity in the mold mycelium was observed as early as in the period of preliminary cultivation, while, under normal conditions of fermentation, the activity of the enzymes should be insignificant during that period. The pH value of the culture medium, for days after inoculation into the large tank, dropped down to 4.35-4.50 in this experiment, together with a gradual increase to the alkaline side up to pH 7.8.

Peroxidase and catalase activity in the culture fluid, when fermentation was impaired on the 5th day, proved to be only half the norm. As a result of these disturbances, penicillin yield was five times lower than the yield obtained under normal conditions of cultivation.

It may be inferred that the intensive activity of peroxidase and catalase in the mycelium grown for inoculation appeared as a consequence of inconspicuous disturbances in the aeration of the medium during the period of early development of the mold. Resulting from that, even greater distortions in the metabolic processes could be anticipated during the succeeding hours of fermentation, in particular impairment of the regular rise in enzyme activity and shift of the activity maximum to a later time, as well as insufficient accumulation of the biomass.

During the same time, the tendency of the culture medium to acidification, manifest as a result of accumulation of a large amount of organic acids and peroxides, had an unfavorable effect in turn on the intracellular metabolism of the mold. Penicillin biosynthesis evidently stopped. The yield of penicillin and also of enzymes from the cell to the culture medium suffered a drop.

Some authors [8] feel that, with the drop in pH value of the medium, there probably takes place a formation of a considerable quantity of organic acids in the penicillin-producing cultures. On the other hand, the inference may be warranted that earlier enhancement of the activity of the mold enzyme (in the inoculum) and insufficiently rapid enrichment of the mycelium by the enzymes during the further course of growth of the mycelium give rise to a noncorrespondence in the synthetic processes, thus retarding penicillin formation.

Thus, in discovering a high peroxidase and catalase activity in the mycelium during the preliminary cultivation, further disturbances should be expected in the enhancement of the activity of the enzymes and in penicillin accumulation.

The disturbances which we observed in the regular activity of the enzymes and the sharp drop in penicillin yield in the culture medium concomitant with it give us grounds for the assumption that penicillin formation in Penicillium chrysogenum Q-176 is directly linked to the oxidative enzyme system, in particular to peroxidase and catalase.

SUMMARY

Studies of peroxidase and catalase in Penicillium chrysogenum Q-176 revealed that peroxidase present in the mycelium is found in a bound state with the cell protoplasm. To determine its true activity, phosphate buffer had to be added to the aqueous mold suspension. Catalase activity in the same suspension was determined without recourse to addition of buffer.

During the growth and development of P. chrysogenum Q-176, peroxidase and catalase activity underwent changes: in the early period of mycelial development, the activity was insignificant and then rose sharply according to the extent of mycelial growth, reaching its maximum and then declining. Parallel to this enhancement of the activity of the enzymes in the mycelium, the activity of the penicillin accumulated in the culture fluid also showed a rise.

When the optimal conditions for cultivation of P. chrysogenum Q-176 suffered impairment, the maximum accumulation of penicillin was shifted to a later period or else its yield was appreciably depressed. Simultaneously, the regular rise in peroxidase and catalase activity in the mold was disturbed. Similar disturbances appeared most often in response to inadequate aeration during the first days of growing the mold or simply in response to shifts of the optimum pH value for the culture medium.

In a mold mycelium grown previously for inoculation, peroxidase and catalase activity was negligible. Where the activity of these enzymes or the activity of one of them showed a significant increase in the inoculum, this led eventually to a disturbance in the regular activity rise in the enzymes in the mycelium, and also to a drop in penicillin yield. Apparently, a close link exists between penicillin biosynthesis and peroxidase and catalase activity in the growing mycelium.

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THE ISOLATION OF A NEW TRANSGLYCOSYLASE-TYPE ENZYME FROM LIVER

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It has been communicated previously from this laboratory that glycogen-like compounds were formed during the incubation of alcohol-treated liver extracts (having no phosphorylase activity) in the presence of glucose [1].

It was shown by these investigations that liver contained the enzyme transglycosylase, which synthesized glycogen-like substances in the presence of α-dextrins and glucose. The present communication deals with the method for the isolation of this enzyme, with its activity and properties.

METHODS

Enzyme Isolation

It was found, as a result of multiple experiments, that the enzyme could be isolated either by ammonium sulfate precipitation at the saturation 0.5-0.7, or by the addition of four volumes of alcohol.

The method finally adopted consisted of the following operations.

The liver was excised immediately after the animal's (rabbit) death and homogenized with an equal volume of ethyl alcohol for 1 min, the precipitated tissue centrifuged off and the alcoholic extract thus obtained was fractionated in the two ways indicated above.

In the first method the extract was diluted with one volume of water, ammonium sulfate was added to 0.5 saturation and the mixture was allowed to stand overnight at 4°. The precipitate was collected by filtration, suspended in a volume of distilled water equal to $^1/_{10}$ of the original (undiluted) extract volume and dialyzed against distilled water at pH 7.5-7.8 for 18-20 hrs. The insoluble residue was discarded. The enzyme solution thus obtained had the appearance of an opalescent, colorless liquid.

In the second method the alcoholic solution was treated with 4 volumes of alcohol and the precipitate thus obtained was dissolved in a volume of water equal to $^1/_{30}$ of the original extract volume; the insoluble residue was discarded, the enzyme reprecipitated with alcohol as above and redissolved in $^1/_{10}$ volume of water (relative to the original extract volume).

Analytical Methods

Synthesized products were determined as follows.

Test solutions, containing the isolated enzyme, were precipitated before and after incubation with trichloroacetic acid and the extract was fractionated with alcohol in the presence of barium, thus yielding a precipitate and an alcoholic extract [2]. The precipitate, containing phosphorus compounds and glycogen, was dissolved in N/10 HCl and aliquots of this solution were hydrolyzed for 3 hrs in 2.2% HCl for glycogen determination.

The difference in reducing value of the dissolved precipitate, before and after hydrolysis, which represented mainly the amount of polysaccharide synthesized, was expressed as mg of glucose, as determined by the method of Hagedorn and Jensen.

The alcoholic extract from the trichloroacetic acid extract fractionation was evaporated to dryness, the residue was dissolved in water and the amount of reduction was determined before and after hydrolysis of the compounds not containing phosphorus [2].

The synthesized products were studied not only by the increase of reducing power on hydrolysis, but also chromatographically and by their color reaction with iodine. The solvent system used for chromatography was a mixture of n-butanol–ethanol –water (4:1:5); spot development was obtained with aniline phthalate.

Liver dextrins were isolated by treating the alcoholic extract with 5-6 volumes of alcohol: the precipitate was washed with alcohol and the dextrins were dissolved in distilled water. A preparation of α-dextrins was obtained by incubating purified samples of liver glycogen with saliva (1:10) over a period of 3-5 days, followed by deproteinization of the incubation mixture and evaporation. In order to remove any free sugars the concentrated solution was fractionated with alcohol in the presence of barium (see above) and the alcoholic extract was discarded; alternately the solution was subjected to fermentation with baker's yeast.

The inactivated liver extract which was employed in some experiments was prepared by repeated (about 10 times) evaporation of the alcoholic extract, deproteinization and, finally, fermentation with baker's yeast.

RESULTS

It was shown in the course of experiments that the prepared enzyme was completely inactive in the presence of glucose alone. The enzyme, irrespective of the isolation method, could be activated by the addition of the inactivated liver extract to the reaction mixture.

TABLE 1

Activity Tests on the Isolated Enzyme Preparation.
Incubation mixture: 3 ml of the enzyme solution, 50 mg of glucose and 2 ml of the inactivated extract. Reduction as mg glucose per ml of the dissolved precipitate solution after acid hydrolysis.

Enzyme + glucose			Enzyme + glucose + + inactivated extract			Enzyme + inactivated extract		
incubation time, min								
0	30	90	0	30	90	0	30	90
Enzyme isolated with $(NH_4)_2SO_4$								
0.340	0.330	0.335	0.350	0.500	0.570	0.320	0.330	0.325
0.248	0.250	0.240	0.250	0.300	0.400	0.230	0.240	0.242
0.140	0.148	0.139	0.148	0.256	0.290	0.130	0.125	0.135
0.300	0.310	0.308	0.310	0.450	0.560	0.305	0.310	0.312
Enzyme isolated with alcohol								
0.140	0.146	0.134	0.150	0.360	0.400	0.160	0.165	0.168
0.306	0.310	0.315	0.310	0.330	0.478	0.310	0.305	0.315
0.270	0.263	0.275	0.280	0.380	0.454	0.265	0.270	0.269
0.250	0.260	0.265	0.240	0.345	0.410	0.235	0.240	0.230

As may be seen from Table 1, increase in reducing power after hydrolysis was found only in the incubation mixtures containing the enzyme, glucose and the inactivated extract. In the two remaining cases, that is, in the presence of either enzyme and glucose, or enzyme and inactivated extract, no increase in reducing power was observed.

A comparison of the two enzyme preparations showed that, in the case of the enzyme precipitated with four volumes of alcohol, a considerable amount of oligo- and polysaccharides was coprecipitated. On the other hand, no such contamination occurred when the enzyme was isolated through an ammonium sulfate precipitation, and the latter method was therefore adopted throughout the subsequent experiments.

It was also found (cf. Table 2) that the optimal pH of the isolated enzyme lay in the region 4.8-5.2. However, another optimal range, at pH 8.5-9.0, was also found. Since enzymatic activity was more pronounced in the range of pH 4.8-5.2, acid conditions were employed throughout, using acetate buffer.

It was found, in the investigation of the time-progress of the reaction, that definite and appreciable changes occurred in comparatively short time intervals, namely, 15-30 min. Consequently, the first aliquot after the beginning of the reaction period was withdrawn at about 20-30 min, and the last one – after 80-90 min.

TABLE 2

The pH Optimum of the Isolated Enzyme.
Incubation mixture: any 3 ml of the enzyme solution + 50 mg of glucose + 2 ml of the inactivated extract. Reduction as mg glucose per ml of the dissolved precipitate solution after acid hydrolysis.

pH of solution	Incubation time, min		
	0	40	90
3.8—4.0	0.458	0.460	0.470
4.8—5.0	0.458	0.708	0.738
6.6—6.8	0.500	0.600	0.450
8.5—8.7	0.440	0.550	0.650

It was also established that the increase in reducing power after hydrolysis was accompanied by a decrease in free glucose in the reaction mixture. In this connection the alcoholic extract of the trichloroacetic acid extract was analyzed as well as the barium precipitate (see methods). Experiments were also carried out with and without the addition of glucose to the incubation mixture.

The results of these experiments have been presented in Table 3, from which it will be seen that, in the presence of glucose, the increase in reducing power in the precipitate was accompanied by the decrease of reduction in the alcoholic extract; moreover, these changes corresponded in degree of magnitude.

TABLE 3

Enzymatic Activity in the Presence and Absence of Glucose.
Incubation mixture: 2 ml of the enzyme solution + 40 mg of glucose + 1.5 ml of the inactivated extract + 0.2 ml of M/5 acetate buffer pH 4.8-5.0 .

Alcoholic extract: Reduction in mg of glucose per ml before and after acid hydrolysis					Precipitate: reduction in mg of glucose per ml after acid hydrolysis				
0 min a	40 min b	90 min c	decrease in reduc. b – a	decrease in reduc. c – a	0 min a	40 min b	90 min c	decrease in reduc. b – a	decrease in reduc. c – a
In the presence of glucose									
7.900	7.600	7.600	—0.300	—0.300	0.500	0.760	0.780	0.260	0.280
8.040	7.800	7.820	—0.240	—0.220	0.246	0.470	0.478	0.236	0.240
8.600	8.431	8.379	—0.169	—0.221	0.300	0.440	0.480	0.140	0.180
10.000	9.608	9.500	—0.392	—0.500	0.507	0.962	1.040	0.452	0.533
In the absence of glucose									
0.080	0.084	0.090	0	0	0.800	0.790	0.820	0	0
0.100	0.120	0.110	0	0	0.400	0.420	0.425	0	0

On the basis of multiple experiments carried out previously in this laboratory with the alcoholic fractionation of trichloroacetic acid extracts of liver [2], which proved that the alcoholic extract contained glucose alone, it is reasonable to assume that, in the present experiments, the decrease of reduction in these extracts was due to the decrease in their glucose content only. This was also confirmed by the fact that the reducing power of these extracts was not affected by acid hydrolysis (cf. right-hand side, Table 3), which showed the absence of polymerization in this fraction.

In parallel experiments which were carried out in the absence of glucose, no alteration in reducing power could be detected in either the precipitate or the alcoholic extract, which contained only small amounts of glucose (lower part, Table 3).

The results thus point to the importance of glucose in the formation of the complex sugars found in the precipitate.

Repeated tests with maltose have led to the conclusion that, in experimental mixtures containing this sugar, enzyme and acetate buffer, no increase in glucose residues took place in the precipitate fraction (Table 4); nor could any changes in reduction be detected in the alcoholic extract (under identical conditions). However, the enzymatic activity of the same preparation could be clearly demonstrated in the presence of glucose and inactivated liver extract (left-hand side, Table 4).

TABLE 4

Enzymatic Activity in the Presence of Maltose.
Incubation mixture for maltose: 3 ml of enzyme solution + 40 mg of maltose + 0.5 ml of M/5 acetate buffer. Reduction in mg of glucose per ml after acid hydrolysis.

Enzyme + glucose + inactivated extract						Enzyme + maltose							
alcohol extract			precipitate			alcoholic extract			precipitate				
incubation time, in min													
0	30	90	0	30	90	0	30	90	0	30	90	180	360
8.640	8.600	8.420	0.560	0.590	0.700	8.32	8.32	8.32	0.094	0.100	0.102	0.104	0.101
8.80	8.380	7.700	0.507	0.962	1.040	8.4	8.38	8.4	0.100	0.108	0.105	0.096	0.100
8.75	8.54	8.56	0.460	0.680	0.670	8.41	8.45	8.41	0.08	0.089	0.085	—	—
8.50	8.22	8.43	0.600	0.894	0.700	8.21	8.28	8.19	0.098	0.100	0.095	—	—

In this case, the reducing power increased in the precipitate and decreased in the alcoholic extract.

The study of the nature of the inactivated liver extract factors, required for the demonstration of the enzymatic activity under investigation, revealed that they were not phosphorus compounds. The amounts of various acid- and alkali-labile phosphorus compounds present in the incubation mixture were not altered during the reaction progress. The obligatory factors in the reaction were shown to be dextrins. Thus, the various dextrin preparations, when added to the reaction mixture instead of the inactivated liver extract, proved to be as efficient as the latter in prompting the increase of reducing power in the precipitate (Table 5). The quantitative increase in glucose residues in the precipitate occurred both in the presence of liver dextrins and in the presence of α-dextrins prepared by α-amylolysis of purified glycogen samples.

TABLE 5

Enzymatic Activity in the Presence of Inactivated Extract and Dextrins.
Reduction in mg of glucose per ml of the dissolved precipitate solution after acid hydrolysis.

Enzyme + glucose + inactivated extract			Enzyme + glucose + liver dextrins			Enzyme + glucose + α-dextrin1			Enzyme + glucose + α-dextrin 2		
incubation time, min											
0	30	90	0	30	90	0	30	90	0	30	90
0.352	0.569	0.596	0.223	0.340	0.392	0.242	0.300	0.450	0.400	0.440	0.840
0.300	0.470	0.520	0.268	0.470	0.560	0.222	0.152	0.350	0.930	1.060	1.160
0.330	0.426	0.526	0.450	0.590	0.590	0.460	0.680	0.660	0.496	0.600	0.610
0.120	0.215	0.250	—	—	—	0.276	0.344	0.390	0.390	0.450	0.600

The substitution of the inactivated liver extracts with pure dextrin preparations allow us to characterize the process taking place during the activity of the enzyme.

It will be seen from the data presented in Table 6, that the amount of polymerized glucose, or polysaccharide, was increased as a result of incubation of the enzyme with dextrins and glucose; this was judged from

the data of reduction, determined before and after hydrolysis. Thus, in Experiment 1, the amount of polysaccharide was increased by 190% in the incubation period, while in Experiments 2 and 3 glucose polymerization, though not as high, was still considerable. The appearance or intensification of the iodine color reaction of the solution (shown in the table by the sign +), observed in this case, also showed the increased polymerization of the polysaccharides present in the solution.*

TABLE 6

Investigation of the Saccharides in the Precipitate Solution

Expt. no.	Incubation mixture	Reduction in mg of glucose per ml									Iodine test		
		before acid hydrolysis a			after acid hydrolysis b			increase in reduction (b - a)					
		0 min	40 min	90 min	0 min	40 min	90 min	0 min	40 min	90 min	0 min	40 min	90 min
1	Enzyme + glucose + + inactivated extract	2.32	2.45	2.3	2.899	3.354	3.452	0.579	0.962	1.040	+	++	+++
2	Enzyme + glucose + + α-dextrins	0.030	0.031	0.035	0.490	0.710	0.690	0.460	0.690	0.655	+	++	+++
3	Enzyme + glucose + liver dextrins	0.132	0.136	0.138	0.532	0.762	0.710	0.400	0.596	0.612	0	+	++

Note: +, ++, +++ denote the intensity of the iodine reaction in increasing order of magnitude.

When the incubation mixtures from Experiments 2 and 3 were examined chromatographically, different pictures were observed: thus, in Experiment 2 the substances found possessed very low mobility, the spots being found in the vicinity of the starting line.

As incubation progressed, even after a short time interval, the corresponding spots increased in size. In the case of Experiment 3, spots appeared, which were not present before the incubation; their R_f values were approximately half that of maltose, which showed that the compounds formed were of lower molecular weight than in the first instance.

DISCUSSION

The investigations showed that liver contained an enzyme which catalyzed the conversion of glucose into complex polysaccharides of the glycogen type. The reaction proceeded in the presence of glucose and dextrins and resulted in the increase in polysaccharide and decrease in free glucose.

This was demonstrated by the fractionation of the trichloroacetic acid extract of the incubation mixture into fractions containing dextrins (in the precipitate) on one hand, and free glucose (in the alcoholic extract) on the other. The quantitative increase in glucose residues in one fraction (precipitate) was concomitant with its decrease in the other (extract). These data supported the view that the enzymatic reaction under investigation was of the transglycosylase type which, as is known, is characterized by the fact, that the transfer of glucose residues during the reaction takes place without the alteration of the total amount of glycosidic residues in the system. Thus, in the process observed above, the increase in amounts of polysaccharide took place at the expense of free glucose.

The fact that the enzyme under investigation was completely inactive in the presence of glucose alone, and that it was activated by the addition of α-dextrins to the incubation mixture, showed that the latter were not merely a reaction cofactor, but that they also acted as substrates in this process. It is known that glucose

* The iodine coloration of branched (as well as linear) polysaccharides is increased with increasing molecular weight [3].

transfer takes place at the expense of splitting "high energy" bonds, which are not present in free glucose. It is quite obvious that the dextrins were the donor-substrates in this reaction, while glucose (and, possibly, other carbohydrates) was the acceptor-cosubstrate.

The reaction, apparently, proceeds in two steps: the cleavage of the original dextrin into fragments and their transfer onto free glucose. It is quite possible that the transfer of glucose residues onto dextrin molecules may also take place in this reaction; it had been, indeed, demonstrated with many enzymatic processes of this type (see below).

It became obvious from investigations into the enzymes of carbohydrate metabolism in the last 10 years, that many of the hydrolytic enzymes, mainly those hydrolyzing disaccharides, viz. invertase, maltase, galactase, etc., were capable of transferring the cleaved-off, "energy rich" sugar residue to organic acceptors (which, in similar reactions, may be carbohydrates), with the formation of oligosaccharides [4]. This afforded a basis to consider these enzymes of the hydrolytic group as transglucosidases, or transglycosylases according to the nomenclature proposed by Hehre [5].

Enzymes of this type are found in many microorganisms – bacteria, molds, yeasts [4], as well as in higher plants [6, 7] and in animal organisms [8, 9].

It has been pointed out by many investigators that amylases are incapable of transferring split fragments onto acceptors other than water (hydrolysis). However, some of the bacterial polyases have been shown to catalyze, apart from straight hydrolysis, the transfer of sugar residues to carbohydrate acceptors, with the formation of polymers of the starch, dextran and levan types.

Giri [8] reported in 1955 the presence in liver of a transglycosylase, which synthesized from maltose the oligosaccharides maltotriose and maltotetrose. These oligosaccharides were formed, according to the author, in incubation mixtures containing the enzyme, maltose and buffer.

Investigations in this laboratory [1], carried out independently from the work of Giri, have shown that the transglycosylase isolated in the present case differed in its activity properties from that described by the latter author. The fact that the enzyme reported here was inactive in the presence of maltose alone (see Table 4), prompted the conclusion that we were dealing with a different enzyme, whose substrate was not maltose, but more complex saccharides.

In this connection it is of interest to mention the work with another enzyme, D-transglycosylase, isolated from potato tubers [10]. The donor-substrate for this D-enzyme were maltodextrins, starting from maltotriose and higher, while glucose, maltose and other compounds could act as acceptors. Careful investigation by the authors failed to reveal maltose as a substrate of the D-enzyme. Enzymatic activity could not be demonstrated in the presence of this disaccharide. The dimensions of saccharides formed depended on the sizes of the original dextrins present; thus, according to the authors, a typical nondialyzing polysaccharide was formed in the incubation in the presence of maltohexose.

It was concluded, as a result of the present investigations and in consideration of the quoted data, that the liver transglycosylase described in the present work resembled in its properties the D-enzyme. This resemblance was best borne out by the fact that both enzymes were inactive with maltose alone and required more complex dextrins as substrates.

The question as to the nature of the bonds which are involved in the transfer catalyzed by the transglycosylase described above, and the size of the starting substrates for the enzyme, remains currently unanswered and will be the subject of further investigations.

SUMMARY

The isolation from rabbit liver of a nonphosphorylating-type enzyme, capable of catalyzing the formation of glycogen-like compounds, has been described. Free glucose and α-dextrins are necessary factors for the reaction, the net result of which is an increase in polysaccharide, and a decrease in free glucose, in the reaction mixture. This was demonstrated by the increase in the number of glucose residues in one fraction (precipitate) of the trichloroacetic acid extract of the reaction mixture, and their concomitant decrease in another fraction (alcohol soluble). It is suggested that the data support the view that the enzymatic process investigated is a transglycosylase reaction, in which the dextrins are the donor-substrate, while glucose is the acceptor-substrate.

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QUANTITATIVE DETERMINATION OF AMMONIA IN PLANTS CONTAINING TANNINS

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In working with tea leaves, we successfuly established that the most widely employed methods for determining ammonia content in plants could yield results entailing substantial error, where the plants had a high tannin content.

The phenomenon is linked to the fact that, in similar cases, the ammonia is restored, during the analysis itself, as a result of oxidation (in alkaline medium) of component parts of the material undergoing analysis. Upon oxidation of tannins by oxygen from the air, a process which proceeds vigorously in the presence of alkali, oxidation products form which in turn are capable of oxidizing a number of substances contained in the plant material. For example, they bring about oxidative deamination of the amino acids to yield ammonia, as demonstrated by Oparin [1] working with chlorogenic acid, a representative of the tannin group:

$$RCHNH_2COOH + \frac{1}{2}O_2 \rightarrow RCHO + CO_2 + NH_3.$$

More detailed reaction schemes involving the oxidation of amino acids by quinones are given in papers by Braunshtein [2], Trautner and Roberts [3], Kursanov [4], Bokuchava and Popov [5].

In all these schemes, the initial step of the oxidative process is the oxidation of polyphenols by oxygen from the air to their corresponding quinones (Fig. 1) which, in turn, brings about through a series of intermediate compounds, the oxidation of amino acids with the formation of ammonia as one of the end products of the reaction.

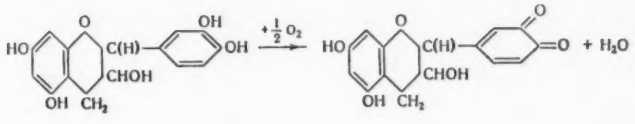

Fig. 1. Formation of quinone on oxidation of catechin.

We observed the restoration of ammonia, on account of the oxidation of amino acids while the analysis was in progress, during ammonia determination in the tea plant, using the microdiffusion method [6].

In order to throw light on the capacity of various polyphenols to serve as hydrogen carriers in the oxidation of amino acids by molecular oxygen in alkaline medium, the following experiment was executed.

50 mg of glycine and individual polyphenols in 100 μmole aliquots were introduced into the outside chamber of a Conway dish, and 2 ml of saturated K_2CO_3 solution was then added. The inner chamber contained 2 ml 0.02 N H_2SO_4. The temperature was 30°, duration of the experiment 12 hrs.

The following results were obtained:

Polyphenols	Ammonia, mg
Pyrocatechin	0.75
Pyrogallol	0.24
Gallic acid	0.17
Hydroquinone	0.48
Phloroglucinol	0.80
Resorcinol	0.20
Turkish tannin	0.54
Tea tannin	0.60

It is clear from the data above that individual polyphenols possess varying ability to bring about oxidative deamination of amino acids when they are undergoing oxidation by oxygen from the air in alkaline medium. The most active in this respect are pyrocatechin, phloroglucinol and tea tannin. The ability is much more weakly expressed in pyrogallol, gallic acid and resorcinol.

Since it is possible to encounter a different quantitative ratio of tannins to amino acids in plants, it was of interest to study the formation of ammonia in response to varied quantitative proportions of tannins to amino acids in the outside chamber of the Conway dish (Table 1).

TABLE 1

Effect of Quantitative Proportions of Glycine and Tea Tannin on Formation of Ammonia on Oxidative Deamination in Potassium Carbonate Solution.
Experimental conditions: in outside chamber: tannin (9 or 90 mg), glycine from 0 to 45 mg, 2 ml saturated K_2CO_3 solution; in inner chamber: 2 ml 0.02 N H_2SO_4; 30°; 12 hrs.

Tannin, mg	Glycine, mg	Ammonia, mg
9	1.50	0.010
9	2.25	0.015
9	4.50	0.030
9	9.00	0.080
9	22.50	0.340
9	45.00	0.490
90	22.50	0.428
90	11.25	0.105
90	5.62	0.040
90	2.25	0.010
90	0.00	0.000

TABLE 2

Formation of Ammonia on Oxidation of Various Amino Acids in Potassium Carbonate Solution in Presence of Pyrocatechin or Tea Tannin.
Experimental conditions: in outside chamber: 10 mg pyrocatechin or tea tannin, 500 micromoles amino acid; 2 ml saturated K_2CO_3 solution; in inner chamber: 2 ml 0.02 N H_2SO_4; 30°; 12 hrs.

Amino acids	Ammonia, mg	
	pyrocatechin	tea tannin
Lysine	0.34	0.13
Valine	0.36	—
Serine	0.44	—
Alanine	0.27	—
Tyrosine	0.12	0.03
Glutamic acid	0.10	0.02
Tryptophan	0.75	0.28
Glycine	0.82	0.51
Cystihe	0.50	0.18

The data in Table 1 show that ammonia formation, under the conditions of the method under discussion, proceeds at the most varied quantitative proportions of tea tannin relative to amino acid, and depends on the amount of both substances present. As the results of the succeeding experiment pointed out, the amount of ammonia present also depends on the nature of the amino acid.

Table 2 shows the results of an experiment in which an identical amount of pyrocatechin or tea tannin, and different amino acids in proportions equivalent to their respective molecular weights, were introduced into the outside chamber of the Conway dish.

As may be appreciated from the data in Table 2, particular amino acids differ appreciably from each other with respect to the amount of ammonia evolved under the conditions of the analysis; the highest capacity in that regard is evinced by glycine.

All the data cited above attest to the fact that in order to obtain more precise data in quantitative ammonia assays in plants containing tannins, conditions must of necessity be brought about where the possibility of the oxidation products of tannins accumulating is eliminated. That may be achieved in any of several ways: by removing the tannins from the material to be analyzed, by driving off the ammonia in the absence of molecular oxygen, by adding substances which act as antioxidants for tannins.

In accord with our findings, ascorbic acid is suitable for the role of antioxidant. In a paper by Bokuchava, Popov and Shubert [7], ascorbic acid reduced quinonic forms of tea catechins to unoxidized catechins, itself being oxidized to dehydroascorbic acid (Fig. 2).

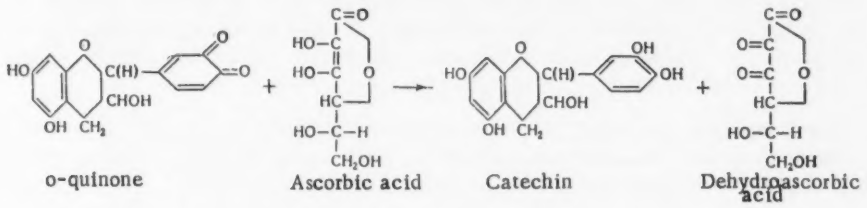

Fig. 2. Reduction of quinone by ascorbic acid.

In order to clear up how complete the antioxidant action of ascorbic acid is with respect to individual polyphenols, and also to establish the required concentration, an experiment was set up in which polyphenols and different amounts of ascorbic acid were introduced into the outside chamber of Conway dishes (Table 3).

TABLE 3

Effect of Ascorbic Acid on Ammonia Formation upon Oxidation of Glycine in Potassium Carbonate Solution in the Presence of Various Polyphenols. Experimental conditions: in outside chamber: 50 mg glycine, 10 mg tea tannin or 10 mg Turkish tannin, 100 micromoles of polyphenols, 2 ml saturated K_2CO_3 solution; in inner chamber: 2 ml 0.02 N H_2SO_4; 30°; 12 hrs.

Ascorbic acid, mg	Ammonia, mg							
	pyro-catechin	pyro-gallol	gallic acid	phloro-glucinol	hydro-quinone	resorcinol	Turkish tannin	tea tannin
0	0.75	0.24	0.17	0.80	0.48	0.20	0.54	0.60
10	0.70	—	—	—	—	—	—	—
25	0.23	—	—	—	—	—	—	—
50	0.11	—	—	—	—	—	—	0.25
100	0.08	—	—	—	—	—	0.00	0.00
200	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

As is clear from the data obtained (Table 3), 0.2 g of ascorbic acid, under the conditions of the experiment, succeeding in blocking the oxidation of all the polyphenols tested, which was accompanied by a complete absence of ammonia formation.

The effective application of the antioxidant action of ascorbic acid was feasible only under the conditions of a closed system, where access was denied to molecular oxygen from the surrounding medium. In the most widespread large-scale method used for the determination of ammonia [6], vacuum distillation is accompanied by the constant access of oxygen from the air through the capillary, and ascorbic acid is rapidly oxidized in that case and is deprived of its antioxidant action. In that connection, the air gaining access to the system via the capillary must be cleared of oxygen, or else an inert gas must be passed.

It must not be overlooked that the data cited represent only a preliminary attempt at perfecting the method of ammonia assay in tannin-containing plants, and it will be desirable to gain further precision in the question touched on in the present discussion.

SUMMARY

The quantitative determination of ammonia in plants having a high tannin content, with the aid of the most widely employed techniques, may yield spurious results due to the reformation of ammonia in the course of the analysis proper.

Tannins, in the presence of alkali, are vigorously oxidized by oxygen present in the air to quinones which in turn elicit oxidative deamination of amino acids present in plant materials, evolving ammonia. The quantity of ammonia produced is dependent on the nature of the polyphenols and amino acids concerned and will be greater as the concentration of these substances in the plant materials increases.

Obtaining more accurate results in the quantitative determination of ammonia in tannin-containing plants may be achieved by several alternative approaches and, in particular, by using, under the given conditions, substances which exhibit antioxidant action with respect to tannins.

I take the opportunity to express my deep-felt gratitude to Professor M. A. Bokuchava for the attention and interest he displayed in the present research.

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A STUDY OF THE PRODUCTS OF HYDROLYSIS OF THE CHAGI COMPLEX ACTING PRINCIPLE USING PARTITION CHROMATOGRAPHY TECHNIQUES

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In studying the chemical composition of chagi, i. e.,of the excrescences formed on birch tree by the mycelia sterila of the pore fungus Inonotus obliquus f. sterili, it was demonstrated that the presence of a dark-colored organic complex, readily soluble in water, was particularly characteristic of chagi [1].

Using as a basis the chemical analysis data from aqueous extracts of chagi, and also the physiological properties of the pigmented complex, the assumption was advanced that this complex is the active acting principle of the medicinal preparation obtained from chagi [2, 3].

The pigmented chagi complex is a high condensation compound of aromatic character and possesses a highly complicated chemical composition. When the complex is digested by 12% hydrochloric acid under pressure, among the products of the hydrolysis, detected by using the method of filter-paper chromatography, were counted four aromatic acids, giving brightly colored characteristic spots on chromatograms with diazotized solutions of n-nitranaline and sulfanilic acid.

In order to shed some light on the nature and makeup of the pigmented chagi complex, it was very important to clear up the nature of those acids, since this might provide a clue as to the character of those more simple compounds which lay at the basis of the condensed chagi pigment under study, and might also point out to what category of natural compounds the complex concerned might most suitably be assigned.

The object of the present discussion was to separate out, in pure form, the aromatic acids referred to from the products of the hydrolysis, and to determine their chemical nature.

We used the method of partition chromatography on silica gel columns to resolve the problem.

EXPERIMENTAL

1. Obtaining the Fraction of Aromatic Acids from Hydrolyzates of the Complex Pigmented Chagi Complex

A precipitate, thrown down from aqueous extracts of chagi on acidification with HCl, was subjected to hydrolysis. The hydrolysis was carried out using 12% HCl, in an autoclave, at 1.5 atmos pressure for 2 hrs. On completion of hydrolysis, the sample, consisting of a liquid portion and an insoluble precipitate, was filtered off, the precipitate was was washed with distilled water and the washings were combined with the liquid portion of the hydrolyzate. To isolate the fractions containing organic acids from the hydrolyzate, the latter was treated several times with ethyl ether. The ethereal extract was then shaken with a 2% soda solution, the aqueous soda layer was separated from the ether, acidified with HCl and acids liberated again transferred to the ether.

Chemical analysis showed that the products of the hydrolysis of the pigmented chagi complex, soluble in ether, consisted of 98% of substances of acidic character, only 2% being assignable to phenols and neutral substances.

2. Study of Ether Extract by Paper Partition Chromatography Method

The qualitative makeup of the ether extract isolated from the hydrolyzate was investigated by the method of paper partition chromatography.

No. 2 and No. 4 grades of chromatographic filter paper manufactured at the Leningrad Volodarski paper factory were used in the analysis. For the mobile solvent phase, several systems of organic solvents were used, of those recommended in the literature for the separation of mixtures of aromatic acids and of some compounds of a phenolic character. In our experiments, the best results in separating out the acids of the ether extracts of the hydrolyzate were achieved in using the solvent system: chloroform–acetic acid–water (50 : 25 : 25) [4]. The method of ascending paper chromatography was used. The chromatographic process was considered completed when the solvent phase had travelled a 22-25 cm distance. The prepared chromatograms were dried overnight at room temperature and then first sprayed with solutions of diazotized sulfanilic acid or n-nitraniline, then with 20% soda solution. An ammonia solution of silver nitrate was also utilized as developing agent.

TABLE 1

R_f Values and Color of Aromatic Acid Spots on Chromatograms of Hydrolyzate Ether Extract. Color developed by diazo reagents and $AgNO_3$ solution.

No. of spot	R_f	Color of spot		
		n-nitraniline	sulfanilic acid	$AgNO_3$
1	0.40-0.50	Reddish-pink	Yellow	–
2	0.70-0.80	Violet	Orange	Dark on heating
3	0.80-0.88	Deep blue	Red	Dark in the cold
4	0.92-0.97	Dingy yellow	Brownish-pink	" " " "

The chromatograms obtained showed that the composition of the ether extracts was very complex: as a rule, no less than 10-12 spots appeared, differing from each other with respect to size, resolution and color intensity. On the basis of the color of the spots, all of them corresponded to aromatic compounds of an acidic character. Especially characteristic of all the chromatograms was the presence of four large spots which gave a specific bright color with diazo reagents.

Table 1 contains R_f values for spots obtained using the solvent system: chloroform–acetic acid–water (50 : 25 : 25), and also the colors of spots developed by diazo reagents and by an ammonia solution of $AgNO_3$.

In order to identify the spots listed in Table 1, we had to isolate the aromatic acids giving those corresponding spots in response to chromatographic treatment, in pure form out of the ether extract, and we then had to determine their chemical nature. For this purpose, as already mentioned above, we used the method of partition chromatography on silica gel columns.

3. Separation of Aromatic Acids from the Ether Extract on Silica Gel Columns

The execution of the work involved was beset with considerable difficulty, inasmuch as the content of the aromatic acids under investigation in the chagi hydrolyzates was not very high and, to isolate them in pure form, large proportions of the fungus had to be treated.

Many methods have been suggested in the literature for effecting the separation of organic acids on chromatographic columns. In the bulk of them, the support columns are prepared using acidic silica gel and butanol–chloroform or amyl alcohol–chloroform systems are used for the mobile solvent phase.

According to data furnished by Moyle and co-workers [5], the sensitivity of the method may be markedly enhanced when silica gel, impregnated with concentrated solutions of phosphate buffer, is used for the separation of a mixture of organic acids. The advantage of this last method lies in the fact that the sample of silica gel and the method of its preparation are without any decisive influence on the degree to which the acids are separated, since that takes place when working with acidic samples of silica gel. Buffer-impregnated silica gel columns were employed by Fischbach et al. for the separation of different types of penicillin [6].

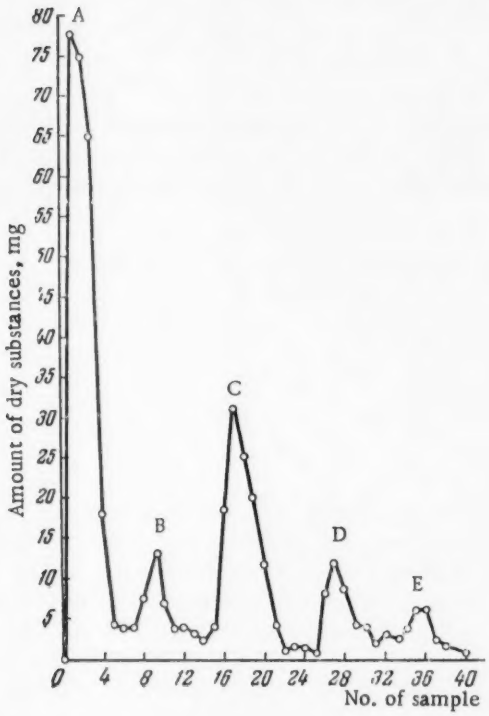

Fig. 1. Separation of aromatic acids of chagi hydrolyzate on silica gel column.

In our experiments, we also used silica gel columns saturated with concentrated phosphate buffers. The silica gel was prepared from commercial water-glass by the method of Gordon et al. [7].

2M KH_2PO_4, K_2HPO_4 and K_3PO_4 were used as buffers, as well as a mixture of these solutions. The mobile solvent phase consisted of a chloroform–butanol mixture. Solutions containing 1%, 10% and 30% butanol in chloroform were used. All of the solutions of butanol in chloroform were saturated before the experiment was started with the corresponding buffer solution.

The course of the analysis was the following. To a silica gel sample was added a buffer solution (to 10 g, 6 ml of buffer) and the mixture was carefully ground in a mortar. It was established by preliminary experiments that the most clearcut separation of the components of the mixture under investigation was observed when the mixture was buffered with a 2M solution of K_2HPO_4. After the silica gel was impregnated with buffer, chloroform was added to it and a column was formed from the whole mixture, in the usual manner. A solution in ether of the acids under study was cautiously poured onto the surface of the prepared column. The column was developed subsequently with 1%, 10% and 30% solutions of butanol in chloroform.

Throughout the development, equal portions of the eluate were collected; depending on the size of the column, the volume of the eluates comprised 10 ml or 20 ml. The complete washing down of the acids under study usually required collecting about 40 samples of the eluate. The amount of the eluted acids present in each sample was determined according to the weight of dry residue of the sample.

The qualitative composition of the eluates was verified via a color reaction with diazo reagents. For this purpose, 10 drops of the eluate were transferred to filter paper in each run, and the filter paper was then sprayed using the corresponding reagent.

The results of the experiments showed that the aromatic acids which we studied, isolated from hydrolyzates of the pigmented chagi complex, can be separated from each other with adequate effectiveness on a column of silica gel impregnated with concentrated phosphate buffer solution at pH ~ 8.0.

Figure 1 shows the distribution curve of the dry residues plotted from the results of one of our experiments, according to individual samples of the eluate. The curve shown in the figure has 5 peaks. The first peak was obtained on developing the column with 1% solution of butanol in chloroform (A), the second and third peaks (B and C) on developing with 10% solution, the fourth and fifth peaks (D and E) using 30% solution.

The color reactions obtained by using diazo reagents demonstrated that the samples corresponding to peak A contained acid 4 (Table 1); in ultraviolet light, all the samples of this segment of the curve fluoresced with a dark brown light. The samples of peak B failed to give a color with diazo reagents and, accordingly, contained none of the acids which we were investigating. The samples of peak C gave a bright blue color response with n-nitraniline, the color corresponding to acid 3; peak D showed violet, corresponding to acid 2, and peak E a pink color, i. e., these samples contained acid 1.

That all the peaks have a symmetric form, are comparatively narrow and show almost no overlapping is clear enought, pointing to the rather well-defined separation of the acids investigated.

In our further work, we pooled all the samples of the first segment of the curve, similarly pooling the samples corresponding to the second, third and subsequent segments, designating them as fractions A, B, C, D, and E. It was necessary to keep in mind, however, that those fractions, together with the acids investigated, contained impurities, which were in the main pigmented substances of oily consistency; these impurities failed to give spots on the paper chromatograms when treated.

The quantitative ratio of fractions A, B, C, D, and E in the hydrolyzates of the pigmented chagi complex proved to be quite constant. We analyzed up to 50 different samples of chagi. The content of individual fractions in these samples, expressed in terms of % dry weight passed through the column, varied over the following ranges:

Fraction	Content of fraction in % dry weight of ether extract
A	24-29
B	4-6
C	12-17
D	4-6
E	~2

It should be noted that the yield of fraction A depended to a great extent on the procedure employed in isolating and treating the hydrolyzate. For example, where there was an increase, during the course of the analysis, in the alkalinity of the medium or in the replacement of hydrochloric acid by sulfuric acid, the content of fraction A dropped to 5-10%. This was usually accompanied by some increase in the content of fraction B. These data support the idea that compounds making up fraction A are very unstable and change readily under the influence of some reagents. In contrast to fraction A, the yields of fractions C, D, and E show no appreciable departure from the limits cited even in the case where the hydrolysis was carried out using not hydrochloric acid, but sulfuric acid or 20% KOH.

4. The Isolation of Aromatic Acids from Fractions in Pure Form and Their Identification

To begin with, the degree of purity of each fraction was verified by the method of paper chromatography. When the fraction to be investigated showed traces of another, closely related fraction, it was subjected to a second chromatographic examination by passing it through a silica gel column. The further progress of the purification depended on the properties of the fraction being investigated.

Fraction A. As is clear from the data cited above, fraction A, from a quantitative standpoint, is the largest, compared to the other fractions. It is of considerable interest, since it exhibits several physiologically active features common to medicinal preparations of chagi (action on enzyme systems, on seed germination, antidote action against poisoning of the organism by heavy metals). This fraction is distinguished by its high oxidation potential. It is characteristic of the fraction in question that its solutions fluoresce in the ultraviolet with a dark brown light and that, when gaseous ammonia is passed through a solution of the fraction, a bright orange precipitate of ammonia salts is thrown down.

Microchromatograms have, however, shown that fraction A is inhomogeneous and is a mixture of not less than three substances. In order to separate out the distinct components of the mixture making up the composition of fraction A, the latter was passed in several runs through a column of silica gel, impregnated this time with a buffer of more alkaline pH (2M K_3PO_4), while benzene was used as the mobile solvent phase. After the described supplementary operation, only one spot was detected, corresponding to acid 4 (Table 1) on the microchromatograms of fraction A.

Upon closer inspection, it turned out, however, that even that spot was apparently inhomogeneous, consisting of two distinct spots superimposed on one another, of which one colored yellow under the influence of a solution of n-nitraniline, the other showing a gray-blue color.

In the course of further analyses undertaken with the object of separating the components which produced those two spots on the chromatograms, it was revealed that, in the process of purification, fraction A underwent a change, its composition becoming not more homogeneous, but more complicated than before. Thus, in performing the microchromatographic analysis of fraction A, subjected to the various operations which accompanied the process of purification, or preserved over a long period on chromatograms together with the two spots corresponding to acid 4, the presence of several additional spots was detected, among them spots typical of the aromatic acids which we discovered in the subsequent fractions (C and D).

It follows from the data cited that the aromatic compound included in the composition of fraction A has a complicated structure, is unstable and decomposes readily. It is possible that the makeup of this compound embraces several aromatic acids, including even the two acids which are found, in free form, in fractions C and D.

A study of fraction A calls for carrying out further investigations, a project which is at present underway in our laboratory.

Fraction B. This fraction contains aromatic acid 3 (Table 1), the presence of which is highly characteristic of all hydrolyzates of the pigmented chagi complex.

In order to effect the isolation of the acid to be investigated from fraction B in pure crystalline form, the fraction in question was subjected to separation on a column of silica gel. The solvent was then completely driven off, under a vacuum, a yellow crystalline precipitate remaining on the bottom of the flask. From this precipitate, acid 3 was isolated in the form of perfectly pure crystals by two procedures: by the method of sublimation and by two-fold recrystallization from glacial acetic acid. A considerable portion of the precipitate decomposed upon sublimation.

The crystals formed as needles, did not dissolve in petroleum ether, dissolved comparatively poorly in water and benzene, and dissolved readily in chloroform, ethyl ether, alcohols, acetone and ethyl acetate. Solutions of the acids, transferred to the paper, fluoresced in the ultraviolet with a dull violet light. The alkaline salts were colored intensely yellow.

Crystals obtained via sublimation had a melting point of 204-205° and a molecular weight of 197-205 (determined according to Rast by fusing with camphor).

On the basis of the molecular weight, melting point and color reaction with the reagents tested, acid 3 corresponded to syringic acid.

Fraction D. In order to isolate acid 2 from fraction D, the latter, as in the case of fraction C, was subjected to repeated separation on a column of silica gel, and was then evaporated under a vacuum to effect the complete removal of any traces of solvent. The precipitate remaining at the bottom of the flask was not crystalline and was contaminated to a much greater extent with extraneous pigmented substances, than was the case in fraction C.

Freeing the precipitate from impurities led to some considerable difficulty, since the pigments contaminating it also exhibited acidic properties and were scarcely distinguishable from acid 2 in their solubility in organic solvents. We were successful in obtaining pure crystals of the acid under study from the precipitate via sublimation. In order to first effect the removal from the precipitate of the bulk of the pigmented substances which were acting to hinder the sublimation process, the precipitate was at first treated with a small quantity of chloroform, then with a mixture of equal volumes of petroleum and ethyl ethers. Acid 2 dissolved in chloroform somewhat less readily than the bulk of the pigments, but it dissolved in the mixture of petroleum and ethyl ether, which failed to dissolve the remaining pigments. After such preliminary treatment, we obtained a light-yellow powder, from which acid 2 was already easily separating out in pure crystalline form with the aid of sublimation. The crystals had the form of fine needles. Their melting point was 206-208° and their molecular weight was 159-166.

On the basis of the melting point and the molecular weight, the acid was judged to correspond to vanillic acid. The identity of acid 2 and vanillic acid was confirmed by the technique of paper chromatography in the presence of an indicator, pure vanillic acid. Two different solvent systems were employed: chloroform–acetic acid–water (50 : 25 : 25) and butanol–acetic acid–water (40 : 10 : 50), and the chromatograms were sprayed with several reagents.

The chromatograms so obtained provided confirmation of the identity of acid 2 and vanillic acid.

Fraction E. Acid 1 was contained in pure form in fraction E, in a slight quantity. This acid was identified with the aid of paper partition chromatography using a suitable indicator.

According to data available in the literature [8], syringic aldehyde, parahydroxybenzoic aldehyde and vanillin are among the products of nitroalkaline oxidation of lignin , detected by the technique of paper chromatography. Since we detected the presence of syringic and vanillic acids in the preparations of the chagi hydrolyzate, then, by analogy with lignin, we expected that the acid contained in the composition of fraction E would turn out to be parahydroxybenzoic acid. Chromatographic analysis of acid 1 in the presence of an indicator, pure parahydroxybenzoic acid, was carried out using three different mobile solvent phases and different developing agents (diazo reagents, an ammonia solution of $AgNO_3$, a solution of $FeCl_3$, a solution of phosphotungstic acid and a 0.04% solution of bromophenol blue) and confirmed the identity of acid 1 and parahydrozybenzoic acid.

SUMMARY

A procedure was developed for the chromatographic separation of aromatic acids of the hydrolyzate of the pigmented chagi complex on a column of silica gel as supporting medium.

Three aromatic acids, isolated from the hydrolyzate of the pigmented chagi complex, were obtained in pure crystalline form and identified: vanillic acid, syringic acid and parahydroxybenzoic acid.

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INCORPORATION OF PHOSPHORUS INTO NUCLEIC ACIDS, FIRMLY AND LOOSELY PROTEIN-BOUND, IN RATS AFTER BURN

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It is known from data available in the literature [1] that burn injuries, both slight ones and injuries involving large areas, have not only a local effect, but bring about a strong over-all reaction on the part of the organism. The outcome of burn sickness depends to a considerable extent on those over-all changes. The study of the general changes ensuing in response to burns is therefore of much theoretical and practical interest.

Burn sickness is accompanied by an impairment in all aspects of metabolism and in particular of protein metabolism. The most characteristic sequels of burn-induced changes in protein metabolism are hypoproteinemia, setting in as a consequence of increased protein degradation, plasma losses and slowing up of protein synthesis.

The vicissitudes of protein metabolism in burn sickness have received comparatively adequate study, while the study of nucleoprotein metabolism has been inadequate, although its significance in the vital life processes of the organism is of considerable scope.

From the literature, we are aware of only two investigations taking up the question of nucleoprotein metabolism in burn sickness. Thus, Roht [2], bringing about moderate burns in rats, detected some degree of increase in the incorporation of P^{32} into kidney and spleen nucleic acids, and a depression of this process in the liver, and especially in the intestines. Beliaev [3] observed, in adult guinea pigs 15 hrs after burn trauma, a decrease in the desoxyribonucleic acid (DNA) level and a comparatively small increase in ribonucleic acid (RNA) in comparison with the control.

The authors mentioned studied the change in the metabolism of nucleic acids in response to burn sickness without taking into account the character of the nucleic acid-protein bond. In recent years, Belozerskii [4], Manoilov [5] and others have established the existence of two types of linkage binding nucleic acids to protein: a labile salt linkage and a firm bond. The biological role of the firmly and loosely bound nucleic acids has not been studied extensively, but there is ground for inferring that it is different in each case (Manoilov and Orlov [6]).

Taking the above as a point of departure, it was of interest to study not only the metabolism of RNA and DNA in burn sickness, but also changes in the character of the nucleic acid-protein bond, i. e., the firm and labile linkages.

We began the investigation of this question with the study of the rate of incorporation of phosphorus into firmly and loosely protein-bound nucleic acids.

MATERIALS AND PROCEDURE

The research was carried out on 60 white rats of the same sex, weighing 200-260 g (33 experimental and 27 control animals). The experimental and control animals were all in the same physical state. The burns were applied by bringing a metallic cube block, heated to 100°, to the carefully shaved lateral surface of the trunk of the animals, for one minute. The surface of the burn area was always 9 cm^2, which amounted to 3-6% of the total body surface.

Radioactive phosphorus was injected subcutaneously in the form of 1 ml of a solution of disubstituted sodium phosphate, activity 100 μC, amount of carrier 0.2-0.6 mg. Two hours after introduction of P^{32}, the animals were sacrificed by decapitation, liver, kidney, small intestine and spleen tissues being taken for investigation.

The incorporation of phosphorus into firmly and loosely protein-bound kidney and liver nucleic acids took place directly after the burn was applied, and incorporation into small intestine and spleen nucleic acids took place 24 and 72 hrs after the burn.

Investigation was not carried on to later periods after the burn trauma, since the changes taking place in tissue metabolism at later intervals could be ascribed not to burn, but rather to the presence of a suppurating wound.

TABLE 1

Incorporation of Phosphorus into Nucleic Acids, and Concentration of Nucleic Acids in Organs of Normal Mouse.
Data averaged over 27 experiments.

Factor to be determined	Liver			Kidneys			Small intestine			Spleen		
	RNA	LDNA*	FDNA*	RNA	LDNA	FDNA	RNA	LDNA	FDNA	RNA	LDNA	FDNA
Relative specific activity (RSA)	0.85	0.08	0.40	2.21	0.07	0.48	4.21	1.81	1.88	3.08	2.18	2.30
NA concentration, in g P/g tissue	556	85	89	237	125	104	407	248	213	346	410	288

* LDNA denotes loosely bound DNA.

** FDNA denotes firmly bound DNA.

Separation of the firmly and loosely protein-bound nucleic acids (NA) was carried out on the basis of a procedure developed at our Institute [7]. The principle of the method is as follows: in the presence of NaCl, a firmly bound nucleoprotein and other proteins are precipitated from an alkaline solution by acetic acid, while the loosely bound nucleic acid remains in solution. RNA was separated from DNA using the Schmidt-Thannhauser method. Phosphorus was determined according to Fiske-Subbarow. The NA concentration was expressed in terms of micrograms of nucleic acid phosphorus per 1 g of crude tissue, while the incorporation of phosphorus into the NA was expressed in terms of relative specific activity (ratio of NA phosphorus specific activity to specific activity of tissue inorganic phosphorus).

Table 1 gives the average values for relative specific activity and for the concentration of NA phosphorus in the control rats.

It is interesting to note that the incorporation of phosphorus into loosely bound liver DNA (0.08) and into kidney DNA (0.07) proceeded at a less intense pace than into firmly bound liver DNA (0.40) and kidney DNA (0.48).

The incorporation of phosphorus into loosely bound (1.81) and into firmly bound (1.88) DNA of the small intestine and spleen (2.18 and 2.30, respectively) proceeded at a uniform rate and was considerably higher than was the case for the liver and kidneys.

Incorporation of phosphorus into RNA (bound to protein predominantly by labile linkages) in all organs studied proceeded at different rates: 4.21 for small intestine RNA, 3.08 for spleen, 2.21 for kidneys, 0.85 for the liver.

The phosphorus concentration of small intestine and spleen loosely and firmly bound DNA was lower than in the liver and kidneys. The highest phosphorus concentration in NA was registered in liver ribonucleic acid.

The data we accumulated on changes in nucleoprotein metabolism after burn injury in all organs studied are presented in Table 2.

TABLE 2

Relative Specific Activity and Concentration of Rat Liver, Kidney, Small Intestine and Spleen Nucleic Acid After Burn Injury.
Average data.

Organ	Time after burn	No. of animals	RNA				LDNA				FDNA			
			RSA	RSA in % of stand.	RSA µgP/g tissue	RSA % of stand.	RSA	RSA in % of stand.	content µgP/g tissue	content in % of stand.	RSA	RSA in % of stand.	content µgP/g tissue	content in % of stand.
Liver	Immediately	7	0.89	104	610	109	0.13	163	95	111	0.26	65	99	111
		6	0.37	43			0.04	50						
Kidney	"	7	2.72	123	227	95	0.04	57	115	92	0.80	166	104	100
		6	1.37	62							0.33	88		
Small intestine		13	2.06	48	436	107	0.79	43	240	96	1.05	55	193	90
	In 24 hrs	8	4.72	112	340	83	1.42	78	245	98	1.67	89	185	86
	In 72 hrs	12	3.30	78	406	99	1.37	75	260	104	1.34	71	208	97
Spleen	Immediately	7	4.13	134			2.78	127			2.65	115		
		6	2.40	77	351	101	1.64	75	357	87	1.52	66	325	112
	In 24 hrs	8	2.39	77	325	93	1.79	82	392	95	1.91	83	214	74
	In 72 hrs	12	3.49	113	372	107	3.33	152	429	104	3.45	150	248	86

As is clear from Table 2, the incorporation of phosphorus into liver nucleic acid directly after the burn injury was sustained, compared to the control, showed two patterns. In one group of animals, phosphorus incorporation into RNA showed no change, but rose to 163% for loosely bound DNA. In the second group, there was observed a sharp drop in phosphorus incorporation into RNA (43%) and into loosely bound DNA (50%). The incorporation of phosphorus into firmly bound liver DNA was suppressed to 65% in all the animals. Attention should be given to the fact that the incorporation of phosphorus into loosely bound DNA in 7 animals (out of 13) increased, while incorporation into firmly bound DNA showed a decrease in all cases. The concentration of phosphorus in liver NA evinced a tendency to increase in all fractions.

In the kidneys immediately following burn (Table 2) , in one group of animals, the incorporation of phosphorus into RNA (123%) and into firmly bound DNA (166%) increased, while decreasing in the other group (from 62% to 68%). Incorporation of phosphorus into loosely bound DNA dropped, in all the animals, to 57%. The concentration of kidney NA phosphorus did not change immediately after the burn.

It is fitting to take note that the incorporation of phosphorus into firmly bound DNA increased in 7 animals (out of 13), while incorporation of phosphorus into loosely bound DNA was inhibited in all the animals.

In the small intestine, immediately after the burn (Table 2), a significant drop occurred in the incorporation of phosphorus into all fractions of NA (from 43 to 55%). In 24 hrs, an enhancement of the rate of incorporation of phosphorus into NA took place (incorporation into RNA even exceeded the standard, reaching 112%).

In loosely and firmly bound DNA, these changes were retained for 72 hrs, but incorporation of phosphorus into RNA dropped appreciably (78%). The nature of the suppression and restoration of the incorporation of phosphorus into loosely and firmly bound small intestine DNA was similar.

The concentration of small intestine nucleic acids after burn showed little change (RNA and firmly bound DNA presented an exception, their concentration falling below standard in 24 hrs).

In the spleen, immediately following burn (Table 2), incorporation of phosphorus into NA took on two patterns. In one set of animals, incorporation of phosphorus into all NA fractions was enhanced (from 115 to 134%), in the other, a dropoff (from 66% to 77%) took place. In 24 hrs, all the animals showed an incorporation of phosphorus dropping below the norm, and, in 72 hrs, a sharp stepping-up in the rate of incorporation of phosphorus into spleen NA was observed, this finding particular expression in loosely and firmly bound DNA (150%-152%).

The concentration of loosely bound spleen DNA, immediately after the burn injury, dropped slightly, while firmly bound DNA showed a negligible increase, no change ensuing in RNA. In 24 and 72 hrs after the burn, the concentration of phosphorus in RNA and in loosely bound DNA lay within the standard range, firmly bound DNA being below the standard.

It follows from the data cited that the animals may be divided into two groups on the basis of phosphorus incorporation into nucleic acids immediately after the burn injury was sustained. In one group, together with a depression of phosphorus incorporation, an increase was observed in the rate of incorporation into loosely bound liver DNA, RNA and firmly bound kidney DNA and into all spleen nucleic acid fractions. In the second group of animals, a depression was noted in the incorporation of phosphorus into all nucleic acid fractions of the organs investigated.

Investigations carried out on the small intestine and the spleen at later intervals following burn showed that phosphorus incorporation into all NA fractions of those organs, in 24 and 72 hrs, proceeded in identical fashion.

Our data demonstrate conclusively that nucleoprotein metabolism reacts with great rapidity to a burn injury.

The changes occurring in loosely and firmly bound liver and kidney DNA, immediately after burn, as we established, are probably evidence of a different biological role for those acids.

The different trend observed in phosphorus incorporation into loosely and firmly bound liver, kidney and spleen DNA and into RNA of the same organs immediately after burn is presumably due to a different response of the animal organism.

SUMMARY

Immediately after burn injury, clear-cut changes took place in the incorporation of phosphorus into liver, kidney, small intestine and spleen nucleic acids bound by labile and firm linkages with protein. This shows that nucleoprotein metabolism reacts very rapidly to thermal burns involving even insignificant areas of skin.

Differences were observed in the incorporation of phosphorus into liver and kidney DNA with labile and firm protein linkages, which presumably indicates a different biological role for them.

Immediately following burn injury, phosphorus incorporation into liver RNA and into loosely bound liver DNA, kidney RNA and firmly bound kidney DNA and into all spleen NA fractions proceeded unevenly, being enhanced in some animals and inhibited in the others, apparently a consequence of a different response on the part of the organism of the experimental animals. Phosphorus incorporation into the small intestine NA of all the animals was depressed.

In the small intestine and spleen, 24 hrs after burn, restoration of phosphorus incorporation into NA took place, but in the small intestine it failed to reach the normal level in 72 hrs, although exceeding the normal level in the spleen. Immediately after burn, the concentration of phosphorus in liver, kidney, small intestine and spleen nucleic acids showed little change.

24 hours after burn, the concentration of phosphorus in small intestine RNA and in firmly bound small intestine and spleen DNA dropped. The drop in phosphorus concentration was also observed 72 hrs following burn in firmly bound spleen DNA.

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A COMPOUND COMPLEX ISOLATED FROM THE COELOMIC FLUID OF PUPAE OF THE SILKWORM (BOMBYX MORI)

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In the study of the processes occurring in the course of insect metamorphosis, a great deal of interest attaches to the investigations of the products of both complete and incomplete breakdown of proteins of the larval organs and tissues. Proteins, the basic structural materials, are utilized in the histogenic period for the building of adult insect tissues and the egg development. Papers by many researchers have been devoted to the study of changes occurring in proteins and free amino acids of the hemolymph and of the coelomic fluid during the period of metamorphosis in the mulberry silkworm.

From data obtained by several authors [1-5], it may be inferred that, in response to significant protein changes both in the hemolymph and in the coelomic fluid (the composition of which embraces hemolymph as well as products of lysis), no significant changes were observed in free amino acid content. The quantity of total nonprotein nitrogen (not precipitated by trichloroacetic acid) is subject to changes of a regular nature: it increases during histolysis and decreases with histogenesis. The nature of nonprotein nitrogen, with the exception of free amino acid nitrogen, has not yet been investigated. Some workers assign it to the nitrogen of free polypeptides or of peptides bound to phospholipids [6].

We succeeded in establishing, in studies on protein changes in the coelomic fluid of mulberry silkworm pupae during metamorphosis, that when proteins found in the coelomic cavity fluid are precipitated with 10% trichloroacetic acid, there remains in the filtrate a nondialyzable complex of rather complicated composition; amino acids, carbohydrates and purine bases are detected in its makeup.

The present paper is devoted to the method of isolating this complex and to some features characterizing it.

EXPERIMENTAL

Isolation of the Complex and Methods Used in Investigation

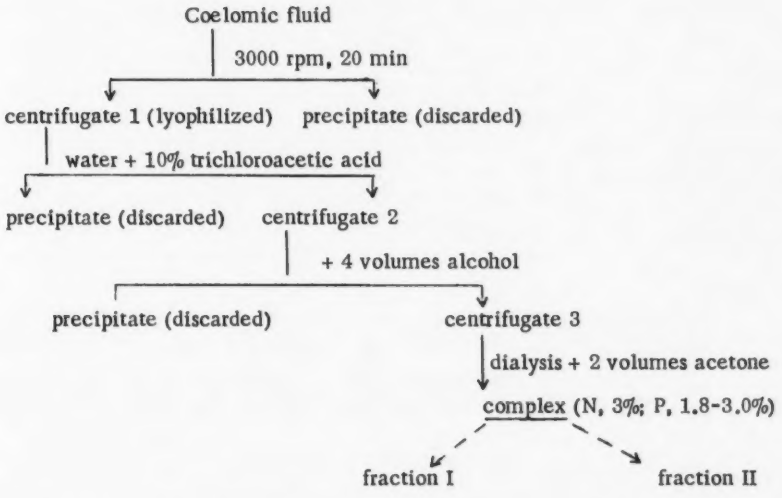
The coelomic fluid from pupae of the "Ukrainskaya" No. 1 variety mulberry silkworm was isolated in a manner described in our preceding article [1], and centrifuged in the cold for 20 min at 3000 rpm. The centrifugate 1 so obtained was lyophilized and served as the material for isolation of the complex.

A certain quantity of the lyophilized centrifugate of the coelomic fluid (2400 mg) was dissolved in distilled water, and an equal volume of 10% trichloroacetic acid was then added to it. Proteins thrown down as precipitate were separated by centrifuging and discarded. Four volumes of ethyl alcohol were added to centrifugate 2 so obtained, and the precipitate, which included glycogen in its composition, was also discarded after centrifugation. Centrifugate 3 was subjected to dialysis for 3-4 days (first in running water, then in distilled water, made alkaline to pH 7-8 by adding 1% NaOH) to remove the alcohol, trichloroacetic acid and dialyzable compounds of the coelomic fluid itself. Dialysis was carried out in the cold. After dialysis was completed, 2 volumes of acetone were added to the solution. The complex, separating out as precipitate, was centrifuged down and lyophilized. The yield of the preparation amounted to 5% of the sample taken. The nitrogen content was 3%, phosphorus content, 1.8-3.0%. The preparation obtained was, in turn, successfully separated into two fractions I and II.

Fraction I was dissolved in distilled water (pH 4.5-5.0) and was successfully separated, by centrifugation, from the water-insoluble fraction II, which remained in the precipitate. Fraction II went into solution only at a pH lower than 3, as well as in different acids (5% trichloroacetic acid, 2N HCl, etc.). The quantity of fraction II comprised 42% of the whole complex.

It must be noted that the precipitation resorted to to isolate the complex, with the aid of acetone, led to splitting off the lipid component and to the breakdown of the complex into low-molecular weight component parts, which already exhibited the property of passing through the pores of a cellophane bag in dialysis. The protracted dialysis probably also resulted in an impairment of the bond linking the individual components of the complex, leading to the breakdown of the latter. All stages of the isolation of the complex are represented in scheme 1 below.

Scheme 1



The amino acid composition of the complex was studied by the chromatographic technique, after acid digestion (6N HCl at 100° for 24 hrs). For the solvent in the chromatographic procedure, a mixture of n-butanol–acetic acid–water (75:7:18) was employed. The chromatograms were developed with 0.2% ninhydrin in acetone and isatine.

By way of investigating its carbohydrate composition, 10 mg of the complex were subjected to hydrolysis by 5% H_2SO_4 for 3 and 5 hrs at 100°. After hydrolysis, the sulfuric acid was neutralized with $Ba(OH)_2$, the solution was evaporated after filtration and transferred to the chromatogram. Solvents used were n-butanol–acetic acid–water (4:1:5) and n-butanol–ethanol–water (40:11:19) mixtures. The solvent was passed over a 76-hr period. The chromatogram, after being allowed to dry, was treated with the developer proposed by Boiarkin [7] which consisted of 0.5 ml orthotoluidine, 0.4 g salicylic acid and 10 ml ethyl alcohol. After drying, the chromatogram was left to stand at 105° for 5 min. Hexoses on the chromatogram were colored green and pentoses red. For chromatographing amino acids and carbohydrates, Leningrad filter paper No. 2 was used. The quantity of hexoses present was determined by the anthrone reaction with boiling for 10 min; the quantity of pentoses was determined according to the orcinol reaction, since, with anthrone, pentoses give a maximum development on boiling for 2 min, and the intensity of the color reaction diminishes considerably if the boiling is continued [8].

The identification of the purine bases was carried out by the chromatographic method after acid digestion by N HCl for 1 hr at 100°, and then later with 72% $HClO_4$, also for 1 hr , at 100° in a sealed ampoule, An n-butanol–water–formic acid (77:13:10) mixture was used. The position of the spots of the purine bases and of the indicators was determined in an ultrachemiscope. For chromatographing, paper which had been previously washed with 15% formic acid and then distilled water was utilized.

The amount of riboflavin present in the complex was determined on the basis of a method advanced for the calculation of bound forms of riboflavin [9].

An electrophoretic study of the complex was carried out in a Tiselius electrophoresis apparatus, in veronal buffer, at pH 5.3 and 7.2, ionic strength 0.1 E = 4 v/cm. A spectrophotometric study of the complex was carried out in a Beckman spectrophotometer, in a 5% $HClO_4$ solution, with no preliminary hydrolysis and with hydrolysis for 15 min at 90°.

Total nitrogen was determined by the Kjeldahl micromethod, phosphorus by the Fiske-Subbarow method. Total, mineral and residual phosphorus were all investigated in the complex. Residual phosphorus was determined on the basis of the difference between total and mineral phosphorus.

Determination of mineral phosphorus was performed in the following manner: 5 mg of the complex were dissolved in 2 ml N HCl at room temperature, and 0.8 ml ammonium molybdate and 0.2 ml eikonogen were then immediately added. The volume was adjusted to 5 ml by adding a solution of N H_2SO_4. After standing for 5 min at 37°, the color intensity was measured on an FEK (photoelectric colorimeter).

EXPERIMENTAL RESULTS

Since the treatment of the complex with acetone led to its splitting up into dialyzable components, the electrophoretic study of the complex had to be carried out before treating it with acetone. Before the experiment, dialysis was carried out for 20-22 hrs to the level of the ionic concentration of the buffer and experimental solution.

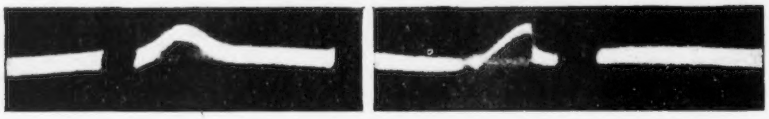


Fig. 1. Electrophoretic pattern of complex untreated with acetone: veronal buffer with pH 7.2, ionic strength 0.1 E = 4 v/cm. Duration of experiment : 12,600 sec.

An electrophoretic curve (Fig. 1) showing one peak was obtained at pH 7.2, the peak having a mobility of $0.4 \cdot 10^{-5}$ cm^2 v^{-1} sec^{-1}. It was noted that the colored portion of the complex, under these experimental conditions, became separated from the basic peak and, being negatively charged, migrated toward the anode, but did not yield an independent electrophoretic pattern. At pH 5.3, the electrophoretic mobility of the peak was $3.5 \cdot 10^{-5}$ cm^2 v^{-1} sec^{-1}.

With the aid of paper chromatography in an n-butanol–acetic acid–water (75 : 7 : 18) solvent mixture, the complex was separated into two spots, which were developed with ninhydrin and gave a phosphorus reaction with Hanes' reagent [10].

Characteristics of Fractions I and II

Fraction I dissolved in water and could be precipitated out of aqueous solution by addition of two volumes of ethyl alcohol. It then precipitated in the form of a gelatinous precipitate. The composition of this fraction, after acid hydrolysis, contained the following amino acids: alanine, phenylalanine, cystine or cysteine, valine, proline, leucine, lysine, tyrosine, arginine, threonine or glutamic acid.

Glucose, mannose and arabinose were successfully identified by the chromatographic method, after hydrolysis, in the carbohydrate component of this fraction. A spot which took on a pink color when developed and which was located above glucose (R_f = 0.09) on the chromatogram, remained unidentified (Fig. 2). On the chromatogram, attention is drawn to the considerable amount of arabinose in comparison to other sugars. In our laboratory, data on the high pentose content in the acid-soluble fraction of the mulberry silkworm pupal coelomic fluid had been obtained previously [4]. The quantity of pentoses in the complex reached 4.0-4.5%, hexoses 2.8-3.0%. It must not be overlooked that a large proportion of the carbohydrates, both hexoses and pentoses, present in the complex are found in the bound state, in fraction I. The polysaccharide in this fraction is precipitated by $Ba(OH)_2$.

Fig. 2. Carbohydrates of the complex: I) unidentified spot (R_f 0.09); II) glucose; III) mannose; IV) arabinose.

Fraction II dissolved well only in acids, while its entering solution was accompanied by a vigorous evolution of carbon dioxide, apparently in the bound state, since repeated reprecipitation of this fraction did not diminish its capacity to liberate carbon dioxide when strong-acid solutions were added. This fraction probably has a large excess of free amino groups, to which the carbon dioxide is bound. In water, this fraction, as has been established, has a pH of 9.4. Haurowitz [11] has pointed out the possibility that carbon dioxide from the air is bound with the formation of carbonates by such alkaline compounds as protamines.

Fraction II contained, for the most part, the same amino acids as were found in fraction I: alanine, cystine or cysteine, tyrosine, valine, phenylalanine, leucine, lysine, arginine, threonine or glutamic acid. After alkaline digestion, tryptophan was not successfully detected by chromatographic methods in either fraction I or II. A qualitative reaction for tryptophan using paramethylaminobenzaldehyde was also negative. No tryptophan was detected in the free amino acids of the silkwork hemolymph [2].

The quantity of carbohydrates in fraction II was considerably less than in fraction I (about 1.4%).

Absorption Spectrum of the Complex in the Ultraviolet

The complex was subjected to spectrophotometric study before being subjected to acetone treatment. In this case, both before hydrolysis using 5% $HClO_4$, and after the hydrolysis, one absorption maximum was detected at 257 mμ and a minimum at 240 mμ (Fig. 3). After being treated with acetone, the complex gave an absorption maximum at 260 mμ only after previous hydrolysis by 5% $HClO_4$. Fraction I revealed, after hydrolysis, an absorption peak at 265 mμ and a minimum at 245 mμ. Fraction II gave two maxima after hydrolysis: one at 255 mμ, the other, less clearly defined, at 270 mμ, with a minimum at 243 mμ (Fig. 4).

The presence of an absorption maximum at 260 mμ, i. e., in the absorption region of nucleic acid components, suggested the possibility of purine bases being present in the complex. After hydrolysis, chromatographic treatment revealed two spots: one corresponding to adenine, the other being apparently guanine. The adenine spot, after extraction with 0.1 N HCl, gave an absorption maximum in the ultraviolet at 260 mμ. It is difficult to specify, as yet, in just what form of combinations purine bases may be found in the complex. Part of the adenine is probably bound to the riboflavin, since it has been determined that the complex exhibits not only absorption in the ultraviolet, but that is also fluoresces in the ultraviolet with a yellow-green color, which is characteristic of riboflavin. The amount of riboflavin in the complex comes to 50 μg/g.

Adenine or its derivatives may be bound to amino groups of amino acids included in the peptides of the complex. Klotz has pointed out the possibility of the formation of such a bond between adenine, adenosine and adenylic acid and proteins (serum albumins) [12].

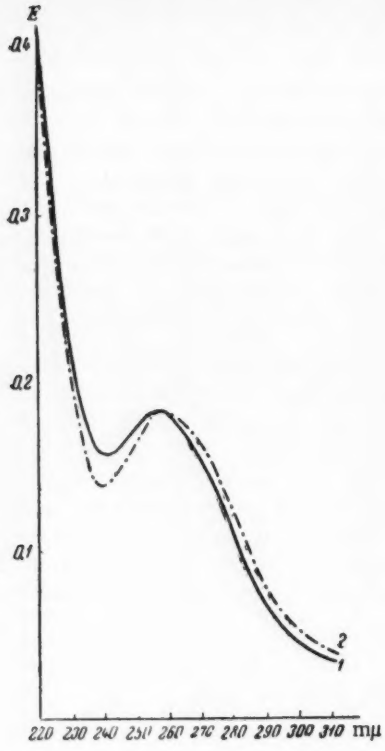


Fig. 3. Absorption patterns of complex not treated with acetone. 1) Before hydrolysis; 2) after hydrolysis with 5% $HClO_4$ at 90° for 15 min.

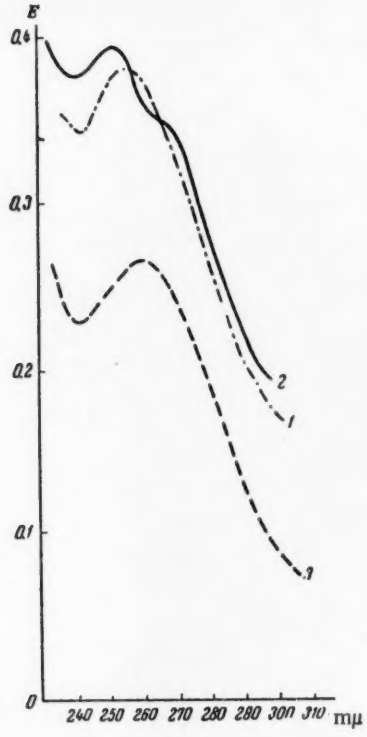


Fig. 4. UV absorption patterns of complex treated with acetone, and of fractions I and II after hydrolysis: 1) complex; 2) fraction II; 3) fraction I. Hydrolysis by 5% $HClO_4$ at 90° for 15 min.

Phosphorus in the Complex

In studying the various forms of phosphorus of the hemolymph and silkworm coelomic cavity fluid and their changes during metamorphosis, a number of investigators [4, 5, 2] have demonstrated that the bulk of acid-soluble phosphorus may be assigned to organically bound phosphorus. The nature of the compounds which contain this phosphorus, aside from glucose-6-phosphate which has been identified, remain unknown.

Phosphorus in Complex and in Fractions I and II

Preparation	Total P in μg/10 mg of complex	Mineral phosphorus		Residual phosphorus	
		in μg/10 mg of complex	in % of total P in complex	in μg/10 mg of complex	in % of total P in complex
Complex	240	79	32.9	161	67.0
Fraction I	150	0	–	150	62.5
Fraction II	90	81	33.8	9	3.7

In fraction II of the complex, phosphorus determined by the Fiske-Subbarow method as orthophosphate, i. e., without previous hydrolysis, was detected. However, paper chromatographic studies established that, in an alkaline solvent mixture of n-butanol–ethanol–NH_3–water (36 : 30 : 10 : 24), the spot corresponding to that phosphorus, in contrast to the orthophosphate, remained at the site of transfer, while, in the acid solvent mixture n-butanol–acetic acid–water (4 : 1 : 5), it traveled 4-5 cm in 48 hrs. When the chromatograms were treated with Hanes' reagent, the spot containing that phosphorus took on a yellow color, which became gray-blue after irradiation with ultraviolet light.

Data on the content of various forms of phosphorus in the whole complex and in its individual fractions are presented in the table above.

The total phosphorus content in the complex, isolated from the miscellaneous starting material, may fluctuate over the range 1.8 to 3.0%. It is clear from the table that fractions I and II differ markedly from one another with respect to the form of phosphorus linkage. Thus, in the water-insoluble fraction II, the main part of the phosphorus (81%) was determined as inorganic phosphorus and comprised 33.8% of the total phosphorus in the complex. The phosphorus present in the water-soluble fraction I was determined only after hydrolysis and amounted to 62.5% of the total phosphorus of the complex. Phosphorus in this fraction is apparently bound to the carbohydrate components of the fraction.

DISCUSSION

The makeup of the mulberry silkworm pupal coelomic cavity fluid contains, along with proteins and amino acids, compound complex substances in which, in addition to amino acids, carbohydrates, purine bases, lipids and riboflavin have been detected.

In the isolated complex, the linkages between individual components are highly labile. Acetone treatment or prolonged dialysis results in destruction of bonds within the complex and in a breakup of the complex into low-molecular weight dialyzable components. Making use of their different solubilities in distilled water, we were successful in separating two fractions out of the complex, each of the two fractions possessing, in turn, a complicated structure. Thus, in fraction I, together with a polypeptide which included a whole series of amino acids in its makeup, a heteropolysaccharide was detected which was in some way bound to the polypeptide, since its precipitation by ethyl alcohol was dependent on the pH of the solution, just as was the precipitation of the polypeptide.

Fraction II turned out to be a polypeptide of alkaline nature whose amino groups probably bind carbon dioxide, as well as compounds of a nucleotide nature, giving rise to an absorption maximum, for the fraction, at 255 mμ. Interest logically centers on the phosphorus present in this fraction, which was determined without preliminary hydrolysis, but was other than an orthophosphate. Being found in fraction II in the bound state, it precipitated from solution in N HCl upon addition of 20% lead acetate, or two volumes of ethyl alcohol. The interpretation of the form in which this phosphorus is present will be the object of further investigation.

The research carried out on the isolated complex compound clear up to some extent the nature of the nonprotein nitrogen, carbohydrates and phosphorus of the acid-soluble fraction of the silkworm pupal coelomic fluid. It is still difficult to say anything definitive as to the physiological role fulfilled by those compounds. Further study of their structure and of the changes which occur during metamorphosis will yield an answer to that question.

SUMMARY

From the coelomic cavity fluid of pupae of the mulberry silkworm, a complex was isolated, containing: nitrogen, 3%; phosphorus, from 1.8 to 3.0%; pentoses, 4.0-4.5%; hexoses, 2.8-3.0%. The complex was divided into two fractions: I and II.

In fraction I of the complex, the following amino acids were detected: alanine, cystine or cysteine, tyrosine, valine, phenylalanine, leucine, lysine, arginine, threonine or glutamic acid; tryptophan was not detected. The carbohydrate component of this fraction contained glucose, mannose, arabinose and an unidentified spot with an R_f of 0.09. The phosphorus in this fraction was determined after hydrolysis and comprised 62.5% of the total phosphorus of the complex.

Fraction II was an alkaline polypeptide bound to a nucleotide component, since the absorption maximum for this fraction lay in the ultraviolet range at 255 mμ. The bulk of the phosphorus present in this fraction was determined as inorganic phosphorus and comprised 33.8% of the total phosphorus of the complex.

Riboflavin was detected in the makeup of the complex in proportions of 50 μg/g.

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THE ACTION OF CHELATING COMPOUNDS ON TISSUE STORAGE AND EXCRETION FROM THE LIVING ORGANISM OF RADIOYTTRIUM, RADIOCERIUM AND PLUTONIUM

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The treatment of toxic effects caused by heavy metals and radioactive isotopes is one of the most complicated problems in therapy. Particular difficulties are presented by the reaction against absorbed portions of metal which gain entry to the blood stream and rapidly become deposited in organs and tissues.

The toxic effect of stable metal isotopes is due, as is known, to the binding cations of the toxic metal acting on free groups of biologically important molecules, or to the displacement of cations present in the latter, which leads to an impairment in the normal functions of those molecules and to a distortion of metabolic processes.

Therefore, it is the task of causal therapy to: 1) avert the absorption of the toxic metal into the blood stream, 2) to speed up the excretion of the absorbed metal from the organism, or 3) to convert the biologically active form of the toxic metal – the cations – into difficultly soluble compounds. The last-named approach must be viewed as palliative, since the metal deposited in insoluble form in the tissues is capable, given some biochemical shifts in the organism, of again converting to an ionogenic state and of producing acute poisoning. In the case of radioactive isotopes, such a method would be generally inapplicable, since the enhancement of tissue storage or the radiating substances would lead to an increase in exposure to radiation.

The methods in existence up to recent times, for enhancing the rate of excretion of toxic metals, aside from their low efficiency, had an important drawback. With the use of those methods, it was possible only to raise the solubility of the difficultly soluble metal compounds deposited in the depot organs, leaving the freed cation a wide range of action, i. e., allowing it the possibility of displaying its toxicity and of becoming again deposited, to a considerable extent, in the organs and tissues.

The advantage of the new chelating principle in the therapy of metal poisoning lies precisely in the fact that we are now in a position not only to dissolve the metal becoming deposited in the tissues, but to deprive its cations of chemical, and consequently, of biological activity as well, by binding these cations in a stable chelate compound which, being highly soluble, is eliminated by the organism in short order, being filtered through the kidneys into the urine. For this purpose, of course, it is required to use such chelates as will yield stable soluble compounds with the metals concerned at the pH level prevalent in the blood, which will not be liable to cleavage under the conditions of the organism's metabolism, and which will, finally, possess a sufficiently low molecular weight to allow their being filtered through the kidneys without hindrance.

Investigations carried out in our laboratory to clarify the possibilities of averting the deposition of metals and radioisotopes in the organism, and also of speeding up the rate of excretion of those portions of metals and radioisotopes already deposited, were begun as early as 1949 and, during the time elapsed since then, an enormous backlog of experimental material has been amassed [1-3].

Research along that line is also of significant theoretical interest, inasmuch as it lays the groundwork for clearing up the role of chelates occurring naturally in the organism, in mineral metabolism.

In the present discussion, the basic results of studies done on different chelates and their effect on the behavior of yttrium, cerium and plutonium in the living organism are presented in concise form. This provides the possibility of comparing the action of various chelates on a given element and the effect of a given chelate on elements exhibiting different patterns of behavior in the organism and different physicochemical properties.

In broad outline, the metabolism of subanalyzable quantities of radioyttrium are characterized by their high extent of accumulation in the skeleton (55-60%), a low degree of deposition in the liver (5-7%) and a rather intense rate of excretion, during the first few days, in the urine (10-15%); plutonium introduced in the form of a citric acid complex, is also deposited preponderantly in the skeleton (55-60%), while it accumulates to an extent of only about 12% in the liver; radiocerium storage occurs, conversely, preponderantly in the liver (45-60%), attaining 25-35% accumulation in the skeleton. The excretion of cerium and plutonium in the urine, during the first few days, does not exceed 2-3% [4].

METHODS

White laboratory rats weighing 150-200 g were chosen as experimental animals. Radioyttrium (Y^{91}) and radiocerium (Ce^{144}), in subanalyzable form (carrier-free), were introduced in the form of chlorides (pH of the solution: 2) into a prepared caudal vein in proportions of 3 mC per rat. Sodium salts of the chelating compounds were injected into the contralateral caudal vein in proportions of $2 \cdot 10^{-5}$ mole (pH 7.3), 2 min after injection of the radioisotope. Plutonium (P^{239}) was introduced intraperitoneally in proportions of 1 mC per rat, in the form of a citric acid complex (pH 6); sodium salts of the chelate were injected into the caudal vein 10 min following the injection of plutonium.

The animals were housed in special cages, allowing the urine and droppings to be collected in each individual case. In the yttrium and cerium experiments, the animals were sacrificed after one day, while, in the plutonium experiment, they were sacrificed four days after introduction of the radioisotope.

For determinations of yttrium and cerium content, the internal organs, as well as the feces, were dried for 5 hrs at a temperature of 150°; the femur and remains of the carcase were ashed in a muffle furnace, and the urine, together with a solution of sodium ethylenediaminetetraacetate (EDTA), which was used to wash down the special cages, was evaporated to a volume of 50-100 ml. From the organs, carcase, femur and feces, ground into a powder, and also from the urine solution, replicate samples were prepared on aluminum cover slides for measurement of radioactivity content, using a B-counter arrangement.

The organs and tissues taken for investigation of plutonium content were wet ashed in concentrated HNO_3 with 30% H_2O_2 added; after ashing, the solutions were evaporated to a dry residue, which was dissolved in a fixed volume of 3N HCl. Replicate amounts of the solutions so obtained were transferred to leaden cover slips of a spherical surface and were dried under an infrared lamp. To effect a more uniform distribution of the sample under study, over the surface of the cover slip a drop of 10% tetraethyleneglycol was added to each sample before drying. The dry residue of the samples prepared in the above fashion did not exceed 0.75 mg per cm^2; as was demonstrated by the measurements carried out, self-absorption of alpha particles was negligible in these circumstances.

The radioactivity of the samples was assayed in an ionization chamber with a linear accelerator and the radioactivity count was taken over the whole organ or over the whole tissue. Following introduction of necessary corrections, the content of the radioisotopes was expressed in terms of percent of the balance, which usually amounted to 96-97% of the radioisotope injected into the rats.

RESULTS

The data presented in Table 1 show that all the chelating compounds tested exert a decisive effect on the storage of yttrium in organs and tissues, as well as on the rate of yttrium excretion in the urine. The most clear-cut rise in the rate of excretion of the radioisotope with the urine was due to uramyldiacetate (UDA) and ethylenediaminetetraacetate (EDTA); there then follow, in order of decreasing effectiveness: α-alaninediacetate (A-1), 1,2-diaminocyclohexanetetraacetate (CH), hexametaphosphate (HMP) and nitriletriacetate (NTA). Inspection of the data in relation to individual organs and tissues shows that the decrease in effectiveness with regard to the elimination of yttrium by the chelates was owing to the less clearly expressed drop in storage of the radioisotope

TABLE 1

Percent Yttrium Content in Organs and Excreta of Rats on First Day of Experiment Under Action of Various Chelating Agents. 5-15 rats to each group.

Chelating agent	Liver	Kidneys	Skeleton	Carcase	Urine	Feces
Control	8.7±0.12	6.8±0.21	42.9±2.14	27.5±1.03	11.6±0.78	2.5±0.11
UDA	1.8±0.50	0.8±0.14	12.8±0.13	1.7±0.67	80 4±0.73	2.5±0.15
EDTA	1.7±0.08	1.0±0.02	10.0±0.73	4.0±0.16	80.3±2.22	3 0±0.16
A-1	3.2±0.50	1.2±0.15	26.6±2.51	10.5±1.53	56.2±0.16	2.3±0.35
CH	4.8±0.24	3.3±0.30	23.1±3.27	17.0±2.25	49.1±2.38	2.7±0.82
HMP	15.3±0.74	3.4±0.21	12.8±1.05	17.3±1.11	48.1±1.64	3.1±0.25
NTA	0.9±0.07	0.4±0.03	35.4±1.25	19.8±0.92	40.8±2.15	2.7±0.13

TABLE 2

Percent Cerium Content in Organs and Excreta of Rats on First Day of Experiment Under Action of Various Chelating Agents. 5-8 rats to each group.

Chelating agent	Liver	Kidneys	Skeleton	Carcase	Urine	Feces
Control	44.8±2.57	2.2±0.51	40.8±2.50	8.5±0.56	3.2±0.25	0.5±0.09
HMP	14.4±1.58	2.5±0.47	20.6±0.78	6.0±1.11	55.0±1.66	1.5±0.21
UDA	16.6±1.57	0.6±0.36	43.7±1.72	3.2±0.91	34.5±2.45	1.4±0.13
A-1	15.5±1.21	0.9±0.27	42.0±2.62	8.0±1.35	32.4±2.33	1.2±0.11
EDTA	18.3±2.28	0.9±0.15	42.1±3.10	6.3±1.40	31.0±1.72	1.4±0.09
CH	23.0±1.41	1.0±0.33	41.2±2.24	5.2±1.12	28.3±0.64	1.3±0.03
NTA	20.5±1.01	1.8±0.23	43.9±2.05	7.0±1.27	25.7±1.34	1.1±0.03

in the carcase on the one hand, on the other (in the case of A-1, CH and NTA) in the skeleton; in the case of HMP, it was due to increased liver storage of the radioisotope.

Sharp differences from the pattern just described may be observed in the cerium experiments (Table 2), where the bulk of the metal eliminated with the urine consisted of a portion deposited in the liver in the absence of any chelate. The order of arrangement of the chelating agents on the basis of their effectiveness is also changed: the greatest rise in cerium excretion in the urine is observed under the action of HMP, sharply lowering the degree of accumulation of the metal not only in the liver, but in the skeleton as well. Differences in the effectiveness of the remaining chelating compounds are to a considerable extent obliterated.

In the experiments with plutonium, two chelating compounds from the aminopolycarboxylic acid group were used: EDTA, as the most effective chelating agent, and NTA, as the least effective in other experiments, as well as a whole group of phosphate chelating compounds, with the aim of clearing up the specific features of their action.

It is clear from the data cited in Table 3 that the most clearly defined drop in accumulation of plutonium in the skeleton, as well as the greatest rise in plutonium excretion with the urine, was brought about by HMP. In addition to that, HMP, along with the other phosphates, somewhat enhanced plutonium storage in soft tissues and soft-tissue organs.* EDTA was inferior to HMP with regard to lessening the degree of deposition in the skeleton and with regard to the total effect of excretion of plutonium from the organism, but did not,however, enhance plutonium accumulation in soft tissues; NTA exerted no statistically reliable effect on the behavior of plutonium in the organism.

* The rise in plutonium storage in the liver ($P = 0.018$) under the action of tetrametaphosphate (from 16.0 to 26.4%) and in the carcase($P < 0.02$) under the action of HMP (from 15.0 to 22.5%) and tetrapolyphosphate (from 15.0 to 21.2%) is statistically reliable.

DICUSSION OF THE RESULTS

In the experiments performed, chelating agents were introduced soon after injection of the radioisotope into the animal (in 2 min, in the case of yttrium and cerium; in 10 min, in the case of plutonium, which was introduced intraperitoneally). In such an experimental arrangement, the chelating ligand encountered the bulk of the incorporated element still in the blood stream and intercellular fluids. A considerable portion of the radioisotope was bound by the chelating partner into a stable soluble compound, in which the metal lost its characteristic properties (specific accumulation in a particular tissue) and was rapidly filtered out through the kidneys.

TABLE 3

Percent Plutonium Content in Organs and Excreta of Rats on Fourth Day of Experiment Under Action of Various Ligands. 5 rats to each group.

Chelating agent	Liver	Kidneys	Spleen	Skeleton	Carcase	Urine	Feces
Control	16.0±0.68	0.9±0.06	0.7±0.05	60.0±1.32	15.0±0.09	1.4±0.42	4.0±0.31
HMP	13.2±2.02	1.7±0.29	1.0±0.28	20.5±2.20	22.5±2.51	30.8±4.86	4.4±0.08
EDTA	12.6±1.02	0.8±0.07	0.5±0.03	40.4±1.51	14.3±0.92	23.3±1.78	5.2±0.37
NTA	14.0±1.01	0.6±0.06	0.7±0.03	52.4±4.21	20.1±2.33	3.4±0.04	5.9±0.66
Orthophosphate	19.0±2.81	1.0±0.07	0.6±0.04	50.7±1.59	18.2±0.95	4.5±0.22	5.1±0.68
Pyrophosphate	17.9±2.47	0.9±0.07	0.8±0.08	50.4±3.05	19.1±0.75	5.1±0.19	4.7±0 31
Trimeta-phosphate	22.9±3.78	1.1±0.03	0.8±0.12	46.3±2.54	17.4±0.36	6.0±0.09	5.0±0.73
Tetrameta-phosphate	26.4±3.38	1.0±0.06	0.6±0.09	45.5±5.60	19.0±2.54	3.1±0.34	4.2±0.55
Tripoly-phosphate	18.1±2.71	1.0±0.10	1.0±0.12	52.8±2.25	19.4±1.35	2.3±0.04	4.6±0.22
Tetrapoly-phosphate	20.3±3.66	0.9±0.07	0.8±0.21	44.2±4.87	21.2±2.27	6.2±0.11	4.6±0.40

In comparing the data obtained, attention centers naturally on the specific group pattern manifested in the action of the ligands. Thus, phosphates (in contrast to other chelating agents) belonging to the aminopolycarboxylic acid compound group, enhance the deposition of yttrium and plutonium in the liver (cf. Tables 1 and 3), and also sharply diminish the storing of cerium in the skeleton, while aminopolycarboxylic acid ligands manifest a directly opposite trend (Table 2). This points out essential differences in the interrelations of the cations and different groups of ligands in the blood stream. At the present state of our knowledge, it is difficult to give an acceptable physicochemical explanation of those differences. Proceeding from the phenomenological picture at hand, it may be assumed that the heightened phosphate concentration which we brought about caused a shift in the usual ratio of physicochemical fractions of metal in the blood; this shift proceeded in the direction of the particles filtering through the kidney membrane (in the case of yttrium and plutonium, mainly on account of the "skeletal" fraction, and, in the case of cerium, also on account of the "liver" fraction), as well as in the direction of the particles taken up by the liver (yttrium and plutonium). It may be inferred that the increased deposition of yttrium and plutonium in the liver, under the action of the phosphates, was due to the formation of difficultly soluble phosphorus compounds taken up by the reticuloendothelial cells. It is known that the solubility of complexed phosphates depends in great degree on the quantitative metal-ligand ratios: soluble phosphates are formed only where an excess of ligand is present. In accordance with that fact, according to our data, a diminution of the dose of phosphate injected leads to an enhancement of yttrium and plutonium storage in soft-tissue organs. It is entirely possible that different quantitative ratios between radioisotope and phosphates are set up in different parts of the organism. The facts disclosed on the one hand bring support to the explanation we advanced, and, on the other hand, are an indication that the overall physicochemical regularities are retained under the conditions of the organism's metabolism.

The aminopolycarboxylic acid group chelating agents yield only soluble complexes under any given conditions, which may shed light on the reasons for the differences observed.

Examination of the data on individual elements indicates considerable departures from the expected relation between the effectiveness of the chelating ligands and the stability constants of the compounds formed with them, on the basis of the hypothesis advanced by us at an earlier date [3, 5]. Thus, the stability constants of

UDA-metal complexes [6] are of an order of 2-3 times lower than is the case for EDTA [7], and 3-4 times lower than for CH [7]; in spite of that fact, UDA complexes are first in the elimination of yttrium and cerium with the urine. Apparently, the stability of UDA-metal complexes is in itself entirely adequate for attaining the maximum effect (elimination of over 80% of the yttrium with the urine). On that basis, it may be viewed that the stability constants of biological metal compounds (for example, metal-bioligand chelates, etc.) lie below the values characteristic of UDA. However, in order to explain the appreciably lower effectiveness of CH (excretion of only 49.1% of the yttrium in the urine), additional factors must be brought into the picture.

Taking into account the complexity of the CH molecule [8], it may be inferred that the orientation of its complexing groups to the metal proceeds at a slower rate, while the rate of complex formation should assume a vital role under the conditions of the living organism, since the ligand and the radioisotope are both rapidly removed from the blood stream and from the intercellular fluids.

The low effectiveness of NTA corresponds fully to the lower stability of the metal complexes formed with it [9], lying at a level of two orders of magnitude lower than the stability constants of complexes formed with UDA as ligand.

It is difficult to give an acceptable explanation for the higher effectiveness of HMP, in comparison with aminopolycarboxylic acid ligands, in the elimination of cerium and plutonium from the organism. The stability constants of its metal complexes are not known, although, judging by the data referable to other metals [10], they should lie to a considerable extent lower than, for example, would be the case with EDTA or CH. The remaining phosphates yield even less stable compounds in combination with cations, which provides an explanation for their low effectiveness in eliminating plutonium.

In comparing the effect of a given ligand (as, for example, EDTA) on the elimination in the urine of the metals under discussion, an order of decreasing effectiveness (on the basis of absolute value) in the following sequence may be noted: yttrium (80.3%), cerium (31%), plutonium (23.3%). This order suggests the dependence of the effectiveness of the ligand on the physicochemical state of the metal in the blood stream. The high level of accumulation of yttrium in the skeleton (55-60%) and the relatively intense rate of excretion of the metal in the urine in the first few days (11.6%) indicates that the bulk of that metal is present in the blood in an ionogenic and molecular-dispersed state (probably a bioligand*) and only an insignificant portion is present in the form of coarsely dispersed colloidal particles, taken up by the liver and by other organs of the recituloendothelial system. This circumstance renders yttrium easily accessible to chelating agents in the blood. The main portion of cerium is deposited in the liver (44.8%; cf. Table 2, "control") and is excreted in the urine in a proportion of only 3.2%, which attests to its coarse state of dispersion in the blood stream; the dissolving of colloidal particles by the action of the ligand proceeds very slowly via the process of peptization, and, during that time, a significant portion of the cerium has time to deposit in the tissues, where it becomes much less accessible to chelating action. We injected plutonium in the form of a citrate complex, which serves to explain its high level of accumulation in the skeleton (60%; cf. Table 3) when compared with a simple salt (as, for example, plutonium chloride) which is deposited predominantly in the liver. However, its unusually low rate of elimination in the urine attests to the fact that the plutonium-citrate complex undergoes rapid breakdown in the blood, and, in taking into account the low pH of the hydrolysis of that element (about 1), it is not difficult to understand that it is quick in forming coarsely dispersed colloidal particles which are difficult for chelating ligands to gain access to.

In conclusion, it is advisable to take note that similar comparative investigations make possible the clarification, with a sufficient degree of precision, of the physicochemical regularities obeyed in the interrelations between cations and chelating ligands under the conditions of the living organism, regularities undoubtedly underlying mineral metabolism. To begin with, the circumstance that, of all the actions tested in our laboratory which exert an effect on the metabolic fate of the incorporated radioisotopes, the greatest effect is that owing to chelating agents, leaves adequate ground for affirming that the bioligands occurring naturally in the living organism assume a most direct role in cation metabolism. To achieve a more profound interpretation of the data cited in the present discussion, a more painstaking study would be required, on the one hand, of the fate of the ligands themselves in the living organism, and, on the other hand, of the stability constants of the compound which they form with the metals under discussion and of the rates of the complexing process.

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* Ligands for yttrium may be furnished by amino acids, peptides, citric acid and a number of other biochelating agents.

From the practical standpoint, such researches would not only shed light on the reasons for the advantages of a particular preparation for medicinal use in treating metal poisoning of the type under discussion, but would also clear the way for organizing, on a more rational basis, further research in developing therapeutic media.

SUMMARY

Chelating agents, binding metal cations to form soluble, stable chelates under the conditions of the living organism, exert a pronounced effect on the radioisotope of yttrium, cerium and plutonium in the living organism. This effect is expressed in the averting of storage of the metals in the tissues and in the enhancement of the rate of excretion of the metal poisons into the urine.

A comparison of the effectiveness of the various chelating ligands assignable to the group of aminopolycarboxylic acid compounds, and also those of the phosphate group, points out substantial differences between the two groups, of both a quantitative and a qualitative nature. The ligands most effective in promoting the elimination of yttrium are uramyldiacetate and sodium ethylenediaminetetraacetate, while the most effective chelating agent acting on cerium and plutonium is hexametaphosphate. The qualitative distinctions relate chiefly to the fact that the phosphates enhance storage of yttrium and plutonium in the tissues, although, in contrast to aminopolycarboxylic acid chelating agents, they sharply reduce the accumulation of cerium in the skeleton.

The data obtained attest to the fact that, aside from the stability of the compounds so formed, essential significance in determining the effectiveness of chelating agents may be assigned to the rate of the complexing process, and also to the physicochemical state of the metal in the blood stream and in the body fluids.

The results cited and the conclusions drawn from them point to a possible role of bioligands occurring naturally in the living organism (amino acids, citric acid, phosphates, etc.) in mineral metabolism.

The high level of effectiveness observed for hexametaphosphate with respect to cerium and plutonium is not susceptible to clinical utilization in view of the considerable toxicity of that preparation. At the same time, the effectiveness of uramyldiacetate, which exceeds that of ethylenediaminetetraacetate, and which lends itself to utilization for the treatment of heavy-metal poisoning and radioisotope poisoning, is deserving of special attention.

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A CONTRIBUTION TO THE QUESTION OF THE MECHANISM OF THIAMINE BREAKDOWN IN THE ANIMAL ORGANISM

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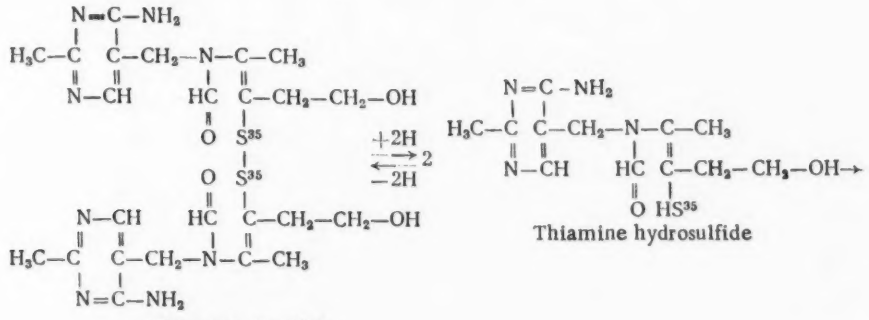
The study of thiamine breakdown in the animal organism, yielding nonvitamin products, is of great interest. It is of especial importance to pay attention to thiamine breakdown in seeking out the reasons for endogenic B_1-hypovitaminoses and in working out reliable methods for determining vitamin B_1 deficiency in the living animal organism.

The study of the metabolic fate of thiamine in the animal organism and, in particular, the study of its breakdown to the resulting end products, has become feasible only with the aid of tracer atoms. S^{35}-tagged thiamine was first obtained by Borsook, Hatcher and Yost [1], who injected it into humans via a parenteral route and established that a considerable portion of the S^{35}-labeled thiamine is destroyed in the living organism down to the sulfate level, the sulfate constituting roughly 25% of all the radiosulfur isolated after injection with thiamine-S^{35}. These data were subsequently confirmed by the present authors [2] in experiments on white mice, guinea pigs and dogs, and were also confirmed by Cerecedo et al. [3] in experiments on white rats. However, the mechanism involved in the oxidation of the thiamine-reduced sulfur to the oxidized sulfur of the sulfates remained obscure.

The present authors demonstrated, in an earlier paper [4], that the oxidation of thiamine sulfur in the living animal organism took place with the participation of amino acids and proteins, which assimilated the reduced sulfur of the thiamine to form sulfur-containing amino acids, which were further oxidized to sulfates.

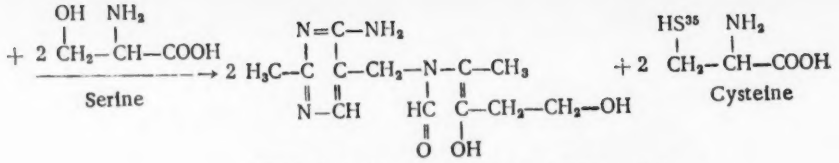
In the present paper, we undertook the study of the effect of sodium hyposulfite, cysteine and ascorbic acid on thiamine breakdown to the sulfate level, in living white rats. It was our view that the above-mentioned reducing agents would contribute to the conversion of thiamine disulfide (an oxidation product of thiamine [5-6]) in living tissues to thiamine hydrosulfide, which would then interact with amino acids and lose its sulfur in the process.

This process may be represented schematically as follows:



Thiamine disulfide

Thiamine hydrosulfide



2'-Methyl, 4'-aminopyrimidyl (5')-methylene-formamino-Δ^3-, 4-methyl, 1,3-dihydroxy-butylene

The cysteine formed thereby is oxidized to sulfates in the living animal organism.

METHODS

Studies were performed on 62 white rats of the same sex (female rats), weighing 200-230 g. The animals studied were maintained, for 5-6 days prior to the experiment and during the entire course of the experiment, on a diet of dry skimmed milk, starch, cod liver oil (in proportions of 4:4:1) and water. Two litter mates of approximately the same weight were selected for each experiment. S^{35}-labeled thiamine was administered subcutaneously to each of them, at a rate of 4000-5000 counts per 1 g of live weight, in doses of 500 μg per animal. In administering the thiamine to the animals, a fixed quantity of nonradioactive thiamine (carrier), equal in weight to the S^{35}-labeled thiamine, was always added such that the total dose of vitamin B_1 administered to the animal would comprise 500 μg in a 0.5 ml volume. Immediately after the thiamine-S^{35} was injected into one of the white rats under study, 50 mg of sodium hyposulfite in a 0.5 ml volume, or 50 mg of cysteine hydrochloride, neutralized with NaOH, or 50 mg of ascorbic acid, neutralized with sodium bicarbonate in the same volumes, were introduced subcutaneously. The rats under investigation were placed on porcelain Büchner funnels and their daily urine was collected.

It was found, in preliminary experiments, that the excretion of radiosulfur in the urine, under the given experimental arrangement, was observable to any significant extent only during the first day after introduction of the S^{35}-labeled thiamine. During the second day, excretion of radiosulfur was 10-15 times lower. Because of that, in all later experiments we investigated only that urine collected in the 24 hrs following the injection of thiamine -S^{35}. Total S^{35} was determined in the urine by ashing according to Benedict [7] and total radiosulfates were determined according to Folin [8]. Both fractions of radiosulfur were isolated in the form of barium sulfate, the activity of which was measured using an end-window counter.

RESULTS

The results of the investigations are presented in Tables 1, 2, and 3, in which the radioactivity is shown for the total S^{35} and radiosulfates in the urine, in counts per min with a correction applied for diuresis. In addition,

TABLE 1

Effect of Hyposulfite on Thiamine-S^{35} Breakdown to Sulfates in Living White Rats

Date of expt.	Radioactivity, cpm scaled to diuresis				% content in urine			
	total S^{35}		$S^{35}O_4^{--}$		"neutral" S^{35}		$S^{35}O_4^{--}$	
	exptl	control	exptl	control	exptl	control	exptl	control
Jan. 21	242970	282310	39000	20720	83.8	92.6	16.2	7.4
Jan. 28	225040	267900	31580	15600	85.7	94.5	14.3	5.5
Feb. 11	262920	281880	40280	9980	84.8	96.5	15.2	3.5
Feb. 18	335280	310800	39600	15040	88.3	95.2	11.7	4.8
Feb. 25	320000	310000	34400	21120	89.3	93.2	10.7	6.8
Feb. 26	254100	283320	27300	15120	89.3	94.6	10.8	5.4
Mar. 2	270000	289110	28170	16500	89.6	94.3	10.4	5.7
Mar. 4	423150	322650	62400	31700	85.4	90.1	14.6	9.9
Mar. 11	304000	273350	45000	12000	85.0	85.6	15.0	4.4
Average:					86.8	94.0	13.2	6.0

TABLE 2

Effect of Cysteine on Thiamine-S^{35} Breakdown to Sulfates in Living White Rats

Date of experiment	Radioactivity, cmp scaled to diuresis				% content in urine			
	Total S^{35}		$S^{35}O_4^{--}$		"neutral" S^{35}		$S^{35}O_4^{--}$	
	exptl.	control	exptl.	control	exptl.	control	exptl.	control
3/19	265395	286665	45162	19343	82.7	93.4	17.3	6.6
3/26	376050	380330	30150	14110	91.9	95.0	8.1	5.0
4/5	331760	280000	30600	13720	90.8	95.1	9.2	4.9
4/9	398720	382280	30480	9880	92.4	97.5	7.6	2.5
4/12	357000	363240	40000	7600	88.6	97.9	11.4	2.1
4/19	337800	321560	33360	16320	89.9	94.9	10.1	5.1
4/21	353400	316710	45460	18930	86.3	93.7	13.7	6.3
4/28	372600	399000	37140	15400	89.7	96.2	10.3	3.8
5/2	373980	364500	51060	19980	86.4	94.5	13.6	5.5
5/9	375900	370920	52000	24800	86.2	93.4	13.8	6.6
5/16	362500	376740	43500	25920	88.0	93.2	12.0	6.8
Average:					88.5	95.0	11.5	5.0

the tables report the relative content (in %) of oxidized sulfate in the radiosulfur and of reduced "neutral" radiosulfur found in the urine. The amount of "neutral" S^{35} is calculated, on the basis of the activity of the total S^{35} and $S^{35}O_4^{--}$ according to the formula

$$\text{Total } S^{35} - S^{35}O_4^{--} = \text{"neutral"} S^{35}.$$

In examining the effect of sodium hyposulfite on thiamine-S^{35} breakdown and on the excretion of total S^{35} and radiosulfates in the urine (Table 1), it should not be overlooked that the excretion of radiosulfates in the urine was enhanced in the experimental animals, while essentially no change was observed in the excretion of total S^{35}. The content of radiosulfates and of "neutral" S^{35} in the urine of the white rats receiving the hyposulfite changed relative to the control rats: the content of radiosulfates was brought up, on an average, from 6.0 to 13.2%, while the content of "neutral" S^{35} dropped from 94.0 to 86.8%.

The increase in thiamine-S^{35} breakdown to the sulfate level, under the influence of sodium hyposulfite, is very clearly expressed. The radiosulfate content of the urine in the experimental animals was higher than in any of the control white rats subjected to study, in spite of a considerable degree of fluctuation in the urine radiosulfate level. On an average, the excretion of radiosulfate in the urine showed an increase of 2.3 times, after the administration of sodium hyposulfite to the white rats.

TABLE 3

Effect of Ascorbic Acid on Thiamine-S^{35} Breakdown to Sulfates in Living White Rats

Date of experiment	Radioactivity, in cmp scaled to diuresis				Content in urine, in %			
	total S^{35}		$S^{25}O_4^{--}$		"neutral" S^{35}		$S^{35}O_4^{--}$	
	exptl.	control	expt.	control	exptl.	control	exptl.	control
5/19	431250	409750	50000	28325	88.4	93.1	11.6	6.9
5/21	439560	452982	32400	21951	92.7	95.2	7.3	4.8
5/28	441100	459680	41800	19680	90.5	95.8	9.5	4.2
6/2	403430	447200	79950	18200	80.1	96.0	19.9	4.0
6/5	458500	499100	46500	15500	90.0	97.0	10.0	3.0
6/7	469160	489440	56240	49680	88.3	89.3	11.7	10.7
6/9	495360	583520	34320	18130	93.2	96.9	6.8	3.1
6/11	505388	539648	45148	11004	91.1	92.5	8.9	7.5
6/14	527000	629820	78675	62440	85.2	90.1	14.8	9.9
6/18	438300	548100	98600	67000	77.6	87.9	22.4	12.1
6/19	431200	609500	85800	42700	88.3	92.9	21.7	7.1
Average:					87.6	93.3	12.4	6.7

The results obtained from studying the effect of cysteine on thiamine-S^{35} breakdown to the sulfate level and its effect on the excretion of total radiosulfur in the urine (Table 2), proved to be in the main similar to the results obtained with sodium hyposulfite. The radiosulfate content in the urine of the white rats to whom cysteine was administered, in comparison to the control rats, rose on an average from 5.0 to 11.5%. A corresponding change was noted in the content of "neutral" radiosulfur in the urine as well.

Thus, cysteine as well as hyposulfite promotes the breakdown of S^{35}-labeled thiamine to the level of sulfates. This enhancement of thiamine-S^{35} breakdown is very clearly pronounced in all the experimental white rats when compared with the control animals; on an average, a 2.3 times increase in thiamine-S^{35} disintegration was observed.

The effect of ascorbic acid on thiamine-S^{35} breakdown in the living organism of white rats, to the level of radiosulfates (Table 3) was in general similar to the effects shown by sodium hyposulfite and cysteine, but was characterized by a few particular features of its own. Among these special features may be considered the effect of ascorbic acid on the excretion of total S^{35} in the urine: in all the experiments, with the exception of one, when ascorbic acid was administered, a slight decrease in the excretion of total S^{35} was registered. This effect caused by ascorbic acid, although negligible, attests to some increase in the deposition of thiamine-S^{35} in the white rat organism. The increase in thiamine storage in the living organism may be explained by the activation by ascorbic acid of enzymes which phosphorylate thiamine to thiamine pyrophosphate [9]. Thiamine pyrophosphate (cocarboxylase) possesses reduced permeability with respect to cell membranes and can to a certain extent be bound to proteins, i. e., to apoenzymes.

The formation of radiosulfates on thiamine-S^{35} breakdown in the living white rat increases in response to the effect of ascorbic acid. However, the effect of ascorbic acid is less clearly expressed than the effect brought about when hyposulfite and cysteine are introduced, due to considerable individual fluctuations in the level of the sulfates present in the urine of the white rats used in the experiments involving ascorbic acid. In the control animals, the content of radiosulfuates in the urine reached 6.7% on an average, and reached an average of 12.4% in the white rats which were administered ascorbic acid. The excretion of radiosulfates in the urine increased 1.8 times on an average after the administration of ascorbic acid to the white rats. A corresponding change was brought about in the content of "neutral" S^{35}.

Summing up the results of the experiments carried out, we came to the conclusion that the reducing agents which we tested (sodium hyposulfite, cysteine and, to a lesser degree, ascorbic acid), when administered to white rats concurrently with thiamine-S^{35}, effected an increase in the breakdown of thiamine to yield radiosulfates in the living organism, the radiosulfates then being eliminated with the urine, in more highly concentrated form. This increase of excretion of radiosulfates in the urine is accompanied by a corresponding decrease in the content of "neutral" S^{35} in the urine of the experimental white rats.

The effect of the above reducing agents on thiamine metabolism brings indirect confirmation to the suggestion advanced at the beginning of the present discussion as to the mechanism of thiamine breakdown in the living animal organism with the participation of amino acids and proteins. The reducing agents administered to the animals increased the concentration of thiamine hydrosulfide in the tissues by reducing thiamine disulfide, and thus stepped up the rate of incorporation of thiamine sulfur into molecules of amino acids and proteins, within the structure of which the sulfur was oxidized to sulfates.

Other possible variants for the pathways of thiamine-S^{35} breakdown in the living organism of rats, under the influence of hyposulfite, cysteine and ascorbic acid, seem less probable to us. In particular, it might be suggested that the reducing agents which we tried out were without effect on the intereaction between the thiamine and amino acids, and promoted rather the oxidation of that sulfur which was of vitamin origin, and which was already taken up into the composition of the amino acids and proteins. To shed light on that suggestion, the corresponding required investigations will be carried out. Still less probable is the assumption as to the effect of the substances experimented with on the excretion of sulfates in the kidneys, since the increase in the content of radiosulfates in the urine of the experimental animals was always accompanied by a corresponding decrease in the content of "neutral" S^{35} in the urine. Excretion of total S^{35} did not increase in the experimental animals in most of the experiments, and even fell off when ascorbic acid was introduced. It must be kept in mind that the elimination of total S^{35} with the urine, after thiamine-S^{35} was introduced, showed a consistent increase in all the rats investigated, with the onset of spring and summer, which might be explained by the extensive saturation of the rat organisms with vitamin B_1 during that period.

SUMMARY

Sodium hyposulfite, cysteine and ascorbic acid enhance thiamine breakdown to yield sulfates in the living organism of white rats.

The stepped-up formation of sulfates from thiamine, under the action of the above-mentioned substances, may be explained by the increased formation of thiamine hydrosulfide in the tissues, this last-named compound interacting with amino acids and proteins to split off the SH-group.

Ascorbic acid, in contrast to hyposulfite and cysteine, increases thiamine storage in living white rats.

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THE BIOCHEMICAL MECHANISM OF ACETYLCHOLINE ACTION

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Clarification of the biochemical nature of the specific sensitivity of effector organs to the mediator of nerve action – acetylcholine – is one of the essential problems of contemporary physiology. Solution of this question would bring the understanding of the effect of cholinergic nerves on organ activity much closer, and would permit interpretation of the mechanism of action of some pharmacological and toxic substances whose action on the animal organism is related to disruption of acetylcholine metabolism.

Data as to the biochemical nature of specific structures of the effector tissue sensitive to acetylcholine – choline receptors, obtained in experiments on frog heart ventricle, are given in the present study.

1. Kinetics of the Interaction of Acetylcholine and Choline Receptors

The high degree of similarity in the kinetics of the most diverse enzymatic reactions, dependent, evidently, upon inherent enzymatic properties which determine the manner of interaction with specific substrates, makes it possible to assume that a similar mechanism of interaction with a specific substrate takes place in nonenzymatic protein, by means of which various nonenzymatic tissue processes are accomplished. Based on this assumption, some attempts were made in the present study, to apply some of the conditions of the kinetic theory of enzymatic processes to the study of the interaction of acetylcholine mediators with specific structures in the effector cells sensitive to this substance, i. e., to choline receptors. Information as to the protein nature of choline receptors was given in our earlier studies [1, 2].

As is known [3-5], the basis of most current theory on the kinetics of enzymatic processes is the idea that enzymatic reactions proceed in two stages: 1) as a result of interaction of the substrate (S) with the enzyme (E), there is formed an intermediate compound (SE), and this reaction is reversible:

$$S + E \rightleftharpoons SE;$$

2) the complex SE breaks down into the free enzyme and the end product of the reaction (P):

$$SE \longrightarrow P + E.$$

The assumption is made that in the interaction of acetylcholine (ACh) with the choline receptor (R), there also takes place the formation of a complex and that the reaction is reversible:

$$ACh + R \rightleftharpoons AChR. \qquad (I)$$

It is assumed that the effectiveness of acetylcholine action depends upon the relationship of the quantity of choline receptors interacting with acetylcholine (AChR) to the total quantity of choline receptors (R), that is, upon [AChR]/[R]. According to the law of mass action the dissociation constant of AChR equals:

$$K = \frac{[ACh]([R] - [AChR])}{[AChR]},$$

where ([R] − [AChR]) is the quantity of free choline receptors not interacting with acetylcholine. From this expression we find that

$$\frac{[AChR]}{[R]} = \frac{[ACh]}{K + [ACh]}.$$

Multiplying both parts of this equation by 100 and setting the left side of the equation as equal to y − the effectiveness of acetylcholine action expressed in percent; it is found that the effectiveness of acetylcholine action is dependent upon its concentration:

$$y = \frac{100\,[ACh]}{K + [ACh]}. \qquad (II)$$

For experimental verification of Expression II we used it after transformation:

$$\frac{1}{y} = \frac{1}{100} + \frac{K}{100} \cdot \frac{1}{[ACh]}.$$

Thus, if Expression II is correct, the relationship of the reciprocal magnitude of effectiveness of acetylcholine action $\frac{1}{y}$ to the reciprocal magnitude of acetylcholine concentration $\frac{1}{[ACh]}$ will be in the form of a straight line, the slope of which is $\frac{K}{100}$, from which it is a simple matter to calculate K.

The experiments were carried out on isolated frog heart ventricles contracted by single stimuli from an induction coil at a rate of 30 stimuli per min. The amplitude of contraction was registered on a smoked kymograph, through use of the usual lever. The acetylcholine solution being studied was prepared in Ringer solution before the test and introduced into the heart ventricle through a cannula. The effectiveness of acetylcholine action (y) was calculated from the expression:

$$y = 100 \cdot \frac{H - h}{H},$$

where H is the amplitude of ventricle contraction under the effect of acetylcholine, and h is the minimal amplitude at the time of the action of the given acetylcholine concentration.

As an example, results of tests with 4 ventricles are given indicating that the effectiveness of acetylcholine action on the contractive properties of heart muscle bears a hyperbolic relationship to the concentration of acetylcholine expressed in g/ml, which corresponds to Expression II (see Fig. 1). The reaction constants correspond to the magnitudes: $K_1 - 3.3 \cdot 10^{-8}$, $K_2 - 1.16 \cdot 10^{-8}$, $K_3 - 0.6 \cdot 10^{-8}$ and $K_4 - 0.3 \cdot 10^{-8}$. Thus the higher the sensitivity of the myocardia to acetylcholine, the lower the dissociation constant of the complex AChR, in which the constant K is numerically equal to the acetylcholine concentration which brings about a 50% depression of the amplitude of cardiac contraction.

2. Effect of High Temperatures on the Condition of Choline Receptors

In this test series the effect of high temperatures (34-40°) on the sensitivity of heart muscle to acetylcholine was studied.

The tests were conducted with isolated frog heart ventricle as follows. After determining the sensitivity of the myocardium to acetylcholine, the ventricle together with the cannula was immersed in Ringer solution, the temperature of which was held at a predetermined level through the use of an ultrathermostat with a precision of 0.1°. Simultaneously with immersion of the ventricle in the solution, liquid was also perfused in the interior

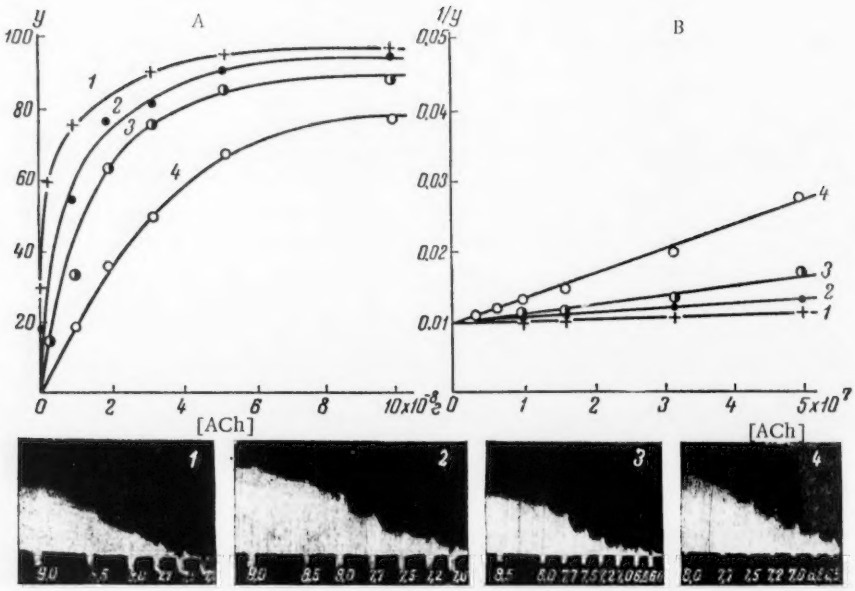


Fig. 1. Relationship of the effectiveness of acetylcholine action on isotonic contractions of isolated frog heart ventricle on the concentration of acetylcholine: kymograms 1, 2, 3, 4) tests on 4 hearts. The figures underneath the kymograms – negative logarithm of acetylcholine concentration. A) Curve calculated from the kymograph data; y) acetylcholine effectiveness in %; [ACh]) acetylcholine concentration (g/ml); B) same data plotted with reciprocal values: 1/y and 1/[ACh].

of the cannula by Ringer solution from the ultrathermostat. After the heat treatment, the cannula with the cardiac muscle was washed externally and internally with Ringer solution at a temperature of 18-20° and the sensitivity of the myocardium to various acetylcholine concentrations was again determined.

First of all, it was observed that the myocardial structures responsible for the accomplishment of acetylcholine action are more sensitive to high temperatures than structures taking part in heart muscle contraction. This circumstance made it possible not to disturb the contractive property of heart muscle, selectively releasing the myocardial sensitivity to acetylcholine.

The degree of heat inactivation of the sensitivity to acetylcholine is extremely dependent both on the temperature and on the length of heating of the ventricle.

Results of experiments are given in Fig. 2A, indicating a decreased sensitivity of the myocardium to acetylcholine when its concentration is $1 \cdot 10^{-6}$ g/ml after treatment of the ventricle with Ringer solution at a temperature of 40, 38 and 37°, depending upon the length of heating. The heart muscle almost completely loses its sensitivity after heating of the ventricle at 40° for 3.5 min. At 38° the loss of sensitivity sets in after uninterrupted heating for 15-17 min. At 37° – after 25-30 min, and at 34° – after 70-80 min of heating.

The data of Fig. 2B show that the effectiveness of the temperature factor decreases considerably if the uninterrupted heating is substituted by intermittent heating. As is seen in Fig. 2A, heating of the heart ventricle at 40° for 3.5 min almost completely ends the sensitivity of heart muscle to acetylcholine. Twofold heating of the ventricle for 2.5 min each lowers the effectiveness of action by 69%. Twofold heating for 2 min leads to a lowering of sensitivity of 35%. A fourfold one-min heating lowers the sensitivity only by 13%, and an eightfold heating for 30 sec produces no change in the sensitivity of myocardium to acetylcholine.

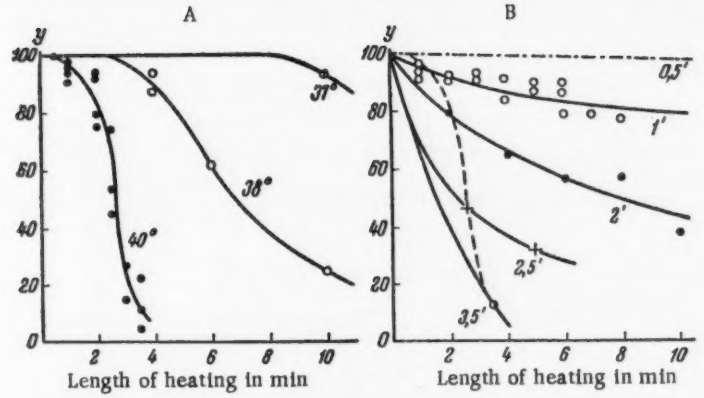


Fig. 2. Changes in effectiveness of acetycholine action on heart muscle in relation to length of ventricle heating: A) decrease in effectiveness of acetylcholine action upon continuous ventricle heating to 40, 38, and 37°. Each point – one test. B) Lowering of the effectiveness of action of acetylcholine after intermittent ventricle heating to 40° for 2.5, 2 and 0.5 min. Each curve – test on one heart.

In these tests, where the action of high temperature was not reversible, the sensitivity of the effector cells to acetylcholine was regenerated, but very slowly – over a period of many hrs, and in some cases there took place only a partial regeneration of sensitivity.

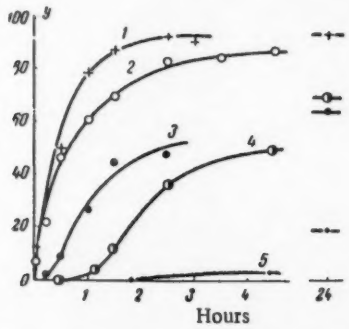


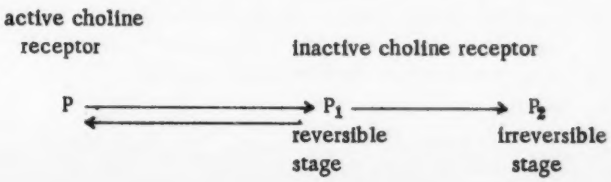
Fig. 3. Regeneration of myocardial sensitivity to acetylcholine ($1 \cdot 10^{-6}$ g/ml) after destructive heat treatment of the ventricle: 1 and 2) after heating the ventricle to 40° for 3.5 min; 3) after fivefold heating of the ventricle to 38° for 6 min; 4) after heating the ventricle for 17 min at 38°; 5) after the heating the ventricle for 110 min at 32.5°.

The curves in Fig. 3 show regeneration of effectiveness of action of acetylcholine after heat treatment of heart muscle. The most frequent irreversible loss of sensitivity is observed upon lengthy treatment of the myocardium at lower temperatures, for example, at 34°. However, in a number of cases and in fourfold action of the effect of the highest temperature, there was observed an irreversible loss in sensitivity of the myocardium to acetylcholine.

Thus upon the action of high temperatures there takes place an irreversible stage of heat inactivation of sensitivity of the myocardial tissue to acetylcholine.

In the next series of tests the effect of multiple heat treatment leading to reversible changes in the sensitivity of heart muscle to acetylcholine (depending upon the effectiveness of action of acetylcholine) to its concentration was studied. First, let us consider the possible mechanism of the change in the kinetics of interaction of acetylcholine with choline receptors upon heat treatment of heart muscle.

Assuming that the decrease in sensitivity of the effector cells upon heat treatment of the ventricle occurs because a portion of the choline receptors go over to an inactive condition:



In this case the dissociation constant of AChR in the interaction of acetylcholine with choline receptors $ACh + R \rightleftharpoons AChR$ will equal:

$$K = \frac{([R] - [AChR] - [R_1] - [R_2])[ACh]}{[AChR]},$$

where $([R] - [AChR] - [R_1] - [R_2])$ is the quantity of free active choline receptors. If the value $\frac{[R_1] + [R_2]}{[R]} \cdot 100 -$ the relative quantity of inactive choline receptors – is designated as n, the effectiveness of acetylcholine action $y = \frac{[AChR]}{[R]} \cdot 100$ will equal:

$$y = \frac{(100 - n)[ACh]}{[ACh] + K}, \text{ or}$$

$$\frac{1}{y} = \frac{1}{100 - n} + \frac{K}{100 - n} \cdot \frac{1}{[ACh]}.$$

Consequently, if the value $\frac{1}{y}$ is plotted against $\frac{1}{[ACh]}$, then upon partial inactivation of the choline receptors the linear relationship of the reciprocal value of the effectiveness of acetylcholine action to the concentration of acetylcholine will be above the normal curve, with the initial point on the ordinate equal to $1/(100 - n)$, and the slope of the curve equal to $K/(100 - n)$.

The results of investigations of the effect of multiple 2-min heating of heart muscle to 40° on the sensitivity of myocardia to acetylcholine and the position of the corresponding kymogram curves are given in Fig. 4.

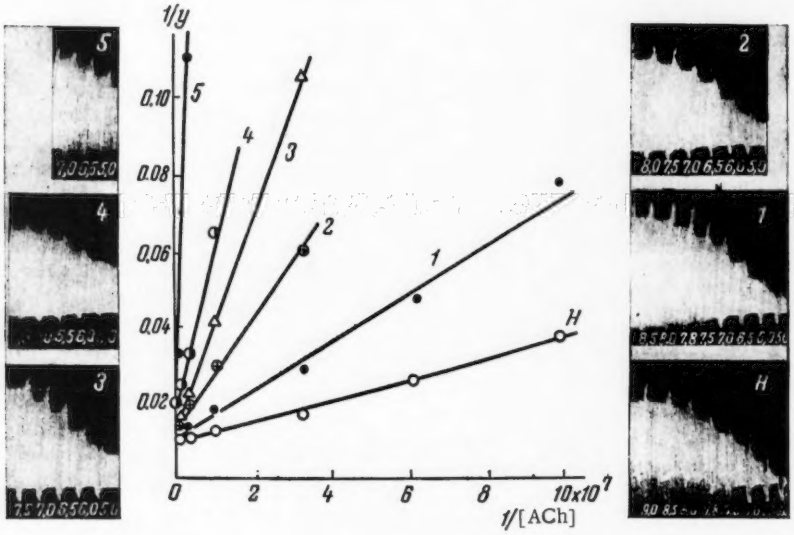


Fig. 4. Effect of multiple heating of heart muscle on the relationship of effectiveness of acetylcholine action to its concentration: H) kymogram and corresponding curve of relationship of effectiveness of acetylcholine action to heat treatment of the ventricle; 1-5) the same after first, second, etc. heating of the ventricle to 40°, each time for 2 min. The figures below the kymogram – negative logarithm of acetylcholine concentration.

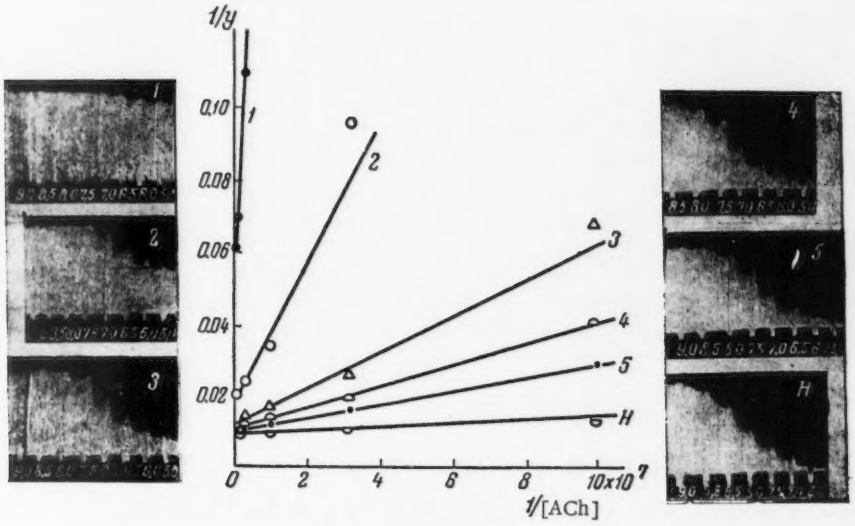


Fig. 5. Regeneration of sensitivity of heart muscle to acetylcholine, which was destroyed by heating the heart muscle to 40° for 3.5 min. H) Kymogram and corresponding curve of relationship of effectiveness of acetylcholine action to heat treatment of the ventricle; 1-5) the same after 5, 30, 60, 90, 120 min after heating. Figures below the kymogram – negative logarithm of acetylcholine concentration.

The magnitudes of K and n, calculated for the curves in Fig. 4, are as follows:

Curve no.	K	n
Norm	$2.7 \cdot 10^{-8}$	0
1	$5.3 \cdot 10^{-8}$	13
2	$1.3 \cdot 10^{-7}$	25
3	$1.9 \cdot 10^{-7}$	33
4	$2.1 \cdot 10^{-7}$	52
5	$7.4 \cdot 10^{-7}$	70

The increase in the value of both constants indicates that the lowering of myocardial sensitivity to acetylcholine proceeds as a result of a decrease in the affinity of choline receptors to acetylcholine, and also as a result of complete inactivation of a portion of the choline receptors and their exclusion from the reaction.

Regeneration of myocardial sensitivity to acetylcholine on the other hand accompanies a decrease in the values n and K, that is, a constant transition of inactivation of choline receptors in an active condition and an increase in the affinity of active choline receptors to acetylcholine (Fig. 5).

For the curves in Fig. 5 the following values for K and n were calculated:

Curve no.	K	n
Norm	$3.5 \cdot 10^{-9}$	0
1	$1.0 \cdot 10^{-6}$	88
2	$0.9 \cdot 10^{-7}$	50
3	$3.7 \cdot 10^{-7}$	22
4	$2.6 \cdot 10^{-7}$	9.0
5	$1.8 \cdot 10^{-8}$	5.0

DISCUSSION OF RESULTS

The data obtained in the present study on the kinetics of the interaction of acetylcholine with effector cells correspond to the data in the literature.

Thus, a similar relationship between the concentration of acetylcholine and effectiveness of action of this substance was found empirically in 1926 by Clark [6] in tests with isometric contraction of heart muscle strips. Clark adduced the following expression:

$$kx = \frac{y}{100 - y},$$

(x – acetylcholine concentration, y – effectivensss of acetylcholine action in %, and k – constant), the identity of which to Expression II shown above is evident, if the Clark expression is solved for y:

$$y = \frac{100\,x}{1/k + x}.$$

The constant k in the Clark expression is the reciprocal magnitude of the constant K of Expression II.

The similarity of Expression II to the expression for kinetics of the enzymatic reaction of Michaelis and Menten, Briggs and Haldane and in many cases with the general theory of Straus and Goldstein [3-5] is evident. There is also identity between the Michaelis constant and the Michaelis and Menten expression and the constant K in Expression II.

The constant K is numerically equal to the acetylcholine concentration which brings about a 50% depression in the amplitude of contraction of the heart muscle, but the reciprocal magnitude of this constant characterizes the degree of affinity of the choline receptor to acetylcholine.

This similarity between the expressions for the kinetics of enzymatic reactions and the kinetics of the interaction of acetylcholine with choline receptors indicates an apparent similarity in the mechanism of formation of complex compounds of acetylcholine with protein – choline receptors and specific substrate of the enzymatic reaction with protein – enzymes.

The tests with heat inactivation of sensitivity to acetylcholine in structures of the myocardium are also very similar to heat inactivation of enzymes, described in the literature (for example, [7]). Also , as in the case of heating of enzymes, upon the heat treatment of the myocardium the choline receptors are converted to an inactive condition in which there take place both reversible and irreversible stages of heat denaturation of the choline receptor.

All the data obtained in the present study, and also the results of previous work, indicate the important role of the SH-groups in the interaction of acetylcholine with effector cells [2], indicating that the choline receptors are protein in nature.

Elucidation as to whether this protein is one of the known enzymes (cholinesterase, as for example, Karasik [8] believes) or an independent protein is to be the object of further study.

SUMMARY

On the assumption that acetylcholine (ACh) exerts its specific action upon the effector organs by interacting reversibly with the choline receptors the following equation has been developed to describe the kinetics of the reaction between acetylcholine and the effector organ:

$$AX + P = AXP.$$

where y is the efficacy of acetylcholine action in %, and K is a constant.

$$y = \frac{100\,[AX]}{K + [AX]},$$

It was demonstrated on an isolated heart ventricle of the frog that the efficacy of acetylcholine action is a hyperbolic function of acetylcholine concentration which is in accord with the above equation.

Exposure of the myocardium to heat (34-40°) resulted in a decrease of the myocardial sensitivity to acetylcholine owing to a reversible and irreversible heat denaturation of the choline receptors. In this case the efficacy of acetylcholine action as a function of its concentration is expressed by the following equation:

$$y = \frac{(100 - n)\,[AX]}{K + [AX]},$$

where n is the relative amount of nonactive choline receptors.

The similarity between the above equations, of acetylcholine interaction with choline receptors and those of the kinetics of enzymatic reactions is considered as a result of similarity in the mechanism of complex compounds formation (of acetylcholine with the protein-choline receptor, on one hand, and of the specific substrates of enzymatic reactions with proteins – enzymes – on the other).

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GLUTATHIONE AND ASCORBIC ACID IN TISSUES OF RATS WITH EXPERIMENTAL HYPOTHYROIDISM PRODUCED BY ADMINISTRATION OF RADIOACTIVE IODINE (I^{131}) AND 6-METHYLTHIOURACIL

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A number of workers have established that radioactive iodine (I^{131}) accumulates in the thyroid when administered to animals and, at a certain concentration, causes destruction of the gland [1]. This enables thyroidectomy to be performed without operative intervention, with formation of a model of experimentally-induced hypothyroidism convenient for the study of thyroid function. Besides radioactive iodine, antithyroid substances such as 6-methylthiouracil (6-MTU) and, lately, potassium perchlorate are used in order to inhibit thyroid function. The possibility is not, however, excluded that these substances exert side-effects in addition to their influence on the thyroid.

In this connection it appeared interesting to compare the results of investigation of metabolism in hypothyroidism produced by I^{131} and by 6-MTU.

The present work is concerned with such a comparison with respect to the influence of the functional state of the thyroid on the metabolism of glutathione and ascorbic acid. Since radioactive iodine and 6-MTU have wide therapeutic application such a study is of considerable importance.

METHODS

Experiments were carried out on male rats weighing 120-200 g. The animals were on the usual laboratory diet consisting of cereal, white bread, milk, meat, and carrots. Experiments were performed on three series of animals: the first series received a single dose (from 0.75 to 1 mC per animal) of I^{131}, the second series received 6-MTU with food in daily doses of 50 mg, the third series received thyroidin in daily doses of 0.1 g per 100 g body weight.

Determinations were carried out at various intervals after administration of I^{131} and 6-MTU. Rats maintained under similar conditions and examined on the same day served as controls.

The free and iron-bound ascorbic acid content was determined by the Tilmans method as modified by Goldshtein and collaborators [2]; glutathione was estimated by the method of Goldshtein et al. [3]. The modification developed by these authors abolished the error caused by the presence of ascorbic acid by means of preliminary oxidation of the acid with ferrous ions.

EXPERIMENTAL RESULTS

Effect on Growth and Development

When radioactive iodine was administered in amounts of 0.75-1 mC, the rats showed lagging in weight, sluggishness, reduced motor activity and aggressiveness. Considerable difference in the weight of internal organs (liver, kidneys, spleen) was noted between the control and experimental animals. There was relative and absolute diminution in the weight of organs in the experimental rats (Table 1).

TABLE 1

Absolute Weight of Viscera in Control Rats and Rats Subjected to Administration of I^{131}

Control						Experimental (I^{131})					
wt. of rat, g	no. of rats	liver in g	kidneys in g	spleen in g	adrenals in mg	wt. of rat, in g	no. of rats	liver in g	kidneys in g	spleen in g	adrenals in mg
100	4	4.30	0.87	0.88	28.7	100	5	3.82	0.79	0.32	18.0
110	2	5.16	0.96	0.85	27.0	110	3	4.00	0.87	0.48	24.5
120	4	4.43	1.08	0.58	24.0	120	3	4.78	0.86	0.50	24.0
130	2	5.28	1.15	0.67	27.0	—	—	—	—	—	—
150	4	5.70	1.25	0.68	27.5	150	3	5.4	1.1	0.63	26.0
190	2	7.57	1.83	1.03	35.5	—	—	—	—	—	—
200	3	8.70	1.88	0.88	34.3	200	2	5.22	1.11	0.47	27.5

As can be seen from Table 1, the greatest difference in weight was noted in the spleen; in a number of experiments the latter's weight was down to one-half or one-third. These data agree with the experimental results of Epel'baum and Dergousova [4] who observed diminution in weight of viscera and especially of the spleen when rats were given 6-MTU. Many authors have observed lagging in body growth and weight of organs in animals subjected to thyroidectomy.

Destruction of the thyroid by radioactive iodine thus exerts the same effect on the growth and development of animals as extirpation of the gland or suppression of its function by means of an antithyroid substance.

Glutathione Content

Our data concerning the glutathione content of normal rat liver are close to the results obtained by other authors using the specific glyoxal method.

According to Schröder and Woodward [5] the glutathione content of normal rat liver is 174-187 mg%. In our experiments the limits of individual fluctuations were wider but the average level of reduced glutathione was 188 mg% (Table 2). Consequently, determination of reduced glutathione with preliminary oxidation of ascorbic acid by ferrous ions gives results which are close to the true content of this substance in the tissues.

TABLE 2

Glutathione Content in Rat Liver in mg%

Preparation administered	Days after admin. of prep.	No. of animals	Glutathione content
Control	—	20	188.8 (131.5—247.9)
I^{131}	20—25	4	284.5 (261—316.2)
"	26—30	4	267.0 (237.6—299.0)
"	32—40	5	258.0 (209.0—332.5)
"	45—50	4	258.5 (236.6—279.5)
6-MTU	23—30	4	358.2 (336.6—396.8)
"	35—40	5	239.3 (224.4—277.4)
"	45—50	5	252.5 (220.0—282.7)
Thyroidin	12--20	4	118.0 (127.5—173.4)
"	25—30	5	145.2 (115.2—190.7)

Note. Limits of fluctuations are given in brackets.

Destruction of the thyroid by radioactive iodine has considerable influence on the hepatic content of reduced glutathione.

Table 2 shows that as soon as 20 days after administration of radioactive iodine the reduced glutathione content in the liver rises. It is possible that the increase in glutathione begins even earlier, since its level was maximal by the time our investigation began. Increased glutathione content persisted throughout the period of experiments which was limited to 2.5 months. No consistent changes could be noted in the glutathione level depending on the duration of I^{131} action.

Increased glutathione content in the liver of rats was also noted when they were given 6-MTU.

When 6-MTU was given in a dose of 10 mg per 100 g body weight no change in the hepatic glutathione content could be seen in three out of four cases; this was evidently determined by small changes in the functional state of the thyroid, reflected in the insignificant degree of its hypertrophy. Administration of 50 mg 6-MTU with the food produced considerable increase

in reduced glutathione content of the liver within three weeks. The glutathione level subsequently declined as a rule, although it remained higher than in normal rats.

The thyroid was markedly hypertrophied in all the rats examined. Its weight in the experimental rats varied from 70 to 194 mg (10-30 mg in normal rats).

The level of reduced glutathione in rat liver thus rises in the presence of hypothyroidism, whether the latter is induced by destruction of the thyroid by radioactive iodine or by administration of 6-MTU.

According to data in the literature, excessive administration of thyroid hormone is accompanied by decrease of glutathione in rat liver and its increase in guinea pig liver [6]. Analogous results (i. e., decrease of liver glutathione content) were obtained by us on feeding thyroidin to rats (Table 2).

The majority of workers deny the presence of oxidized glutathione in the tissues. We detected small amounts of this substance in a number of experiments. However, no consistent changes in the oxidized glutathione content depending on thyroid function could be established. It is possible that these small amounts of oxidized glutathione appear as the result of oxidiation of HS-glutathione in the process of its oxidation determination.

Ascorbic Acid Content

Determination of free and iron-bound ascorbic acid was carried out on liver, kidneys, spleen, brain, testicles and adrenals.

Similarly to the data of other authors [7], we found considerable amounts of iron-bound ascorbic acid in the liver, kidneys and spleen of control animals (Table 3). It was either absent from the brain and testicles or was found in such insignificant quantities that its presence in these organs gave rise to doubts. Bound ascorbic acid was not determined in the adrenals owing to the small amount of material available.

TABLE 3

Ascorbic Acid Content of Organs in Control Rats (in mg%)

Liver		Kidneys		Spleen		Brain	Testicles	Adrenals
free	bound	free	bound	free	bound	free	free	free
25.5	11.0	22.7	4.1	30.0	16.1	41.6	—	—
25.1	7.9	16.9	3.3	32.3	—	33.0	—	—
17.9	6.4	16.9	3.7	23.1	25.5	35.5	—	338
21.3	4.2	15.9	6.7	28.2	15.7	42.5	36.4	300
20.8	11.2	16.6	4.6	39.4	6.3	40.0	28.6	—
22.8	8.3	17.0	4.1	39.8	16.8	—	—	336
24.4	5.0	15.7	3.4	27.6	9.4	38.3	25.5	490
31.1	3.7	18.7	5.3	24.0	9.6	35.8	27.5	373
16.1	9.1	12.6	7.4	19.2	8.8	40.0	24.5	258
20.0	6.7	15.0	5.3	30.0	10.0	42.3	28.2	336
28.5	3.9	19.2	4.1	35.2	6.8	40.0	30.7	396
24.0	—	14.1	—	27.0	—	34.3	24.5	303
Mean: 23.1	7.03	16.7	4.8	29.6	12.5	38.5	28.2	347

No substantial changes in the content of both forms of ascorbic acid depending on the functional state of the thyroid could be discovered. Only a tendency towards diminution of free ascorbic acid could be noted in hypothyroidism; this tendency was particularly evident in the case of liver and testicles (Table 4).

If it is taken into account that the presence of glutathione hinders determination of ascorbic acid and the amount of the former is higher in the liver of hypothyroid rats than in the normal, it is possible that the true amount of ascorbic acid in the liver and possibly other organs of rats may be even smaller. Consequently, it is possible to speak of some reduction in the amount of ascorbic acid in the tissues of hypothyroid animals.

We confirmed the findings of other authors concerning the absence of change in the ascorbic acid content of liver in rats with experimentally-produced hyperthyroidism [6].

TABLE 4

Ascorbic Acid Content (in mg%) in Rat Liver and Testicles in Relation to the Functional State of the Thyroid

Dose	Liver		Testicles	Liver		Testicles	Liver		Testicles
	free	bound	(free)	free	bound	(free)	free	bound	(free)
	Administration of radioactive iodine			Administration of 6-MTU			Administration of thyroidin		
800 μC	19.6	5.9	—	17.3	5.0	23.1	25.5	11.0	—
"	20.4	14.4	—	18.9	6.1	20.9	33.3	9.4	—
"	23.0	9.3	—	13.0	5.5	18.3	18.8	16.5	—
"	18.7	13.1	—	25.0	6.6	23.3	23.1	8.9	21.3
"	15.4	10.2	23.0	26.9	5.9	—	22.5	9.5	21.3
"	17.4	8.3	22.5	24.5	9.3	26.9	22.2	6.5	25.5
1 mC	20.6	15.0	25.8	20.0	10.0	—			
"	19.9	6.9	—	21.8	—	22.4			
				20.7	4.5	—			
				20.0	3.4	27.5			
Mean:	19.4	10.4	23.7	20.8	6.2	23.2	24.2	10.3	22.7

TABLE 5

Ascorbic Acid Content (in mg%) in Rat Organs 20 Hours After Injection of Ascorbic Acid

Experimental conditions	Liver		Kidney		Spleen		Brain	Testicles	Adrenals
	free	bound	free	bound	free	bound	free	free	free
Control	30.0	8.2	22.2	3.9	45.2	4.8	38.2	35.8	416.2
Admin. 6-MTU	25.8	4.2	22.2	3.0	39.0	25.2	36.4	23.1	324.0
Control	26.4	9.4	19.3	1.0	32.0	15.6	37.0	30.0	336.0
Admin. 6-MTU	19.3	3.0	20.5	5.9	41.4	10.8	39.0	27.7	290.0
Control	32.6	4.4	22.6	4.2	46.6	8.8	38.3	34.7	435.1
Admin. 6-MTU	24.4	6.4	22.6	4.2	42.2	15.4	38.3	29.6	406.8
Control	32.0	16.1	21.4	2.4	55.0	—	37.5	38.1	411.0
Admin. 6-MTU	24.0	9.3	20.0	8.9	48.0	13.6	38.6	34.3	266.0
Control	27.9	4.1	15.5	5.7	31.0	22.2	35.8	—	381.0
Admin. 6-MTU	23.6	6.4	18.7	4.4	41.0	12.0	38.0	—	330.0
Control	27.9	4.1	15.5	5.7	31.0	22.2	35.8	28.5	381
Admin. thyroidin	39.6	6.5	24.0	6.0	48.0	9.8	46.0	36.9	451
" "	31.9	9.2	18.2	5.4	32.8	17.2	42.5	38.6	400
Control	26.7	3.1	17.1	5.5	31.0	14.6	35.8	28.2	337
Admin. thyroidin	40.0	5.3	17.1	6.9	51.6	—	34.3	32.8	405
Control	26.5	7.4	21.7	5.4	45.0	12.6	41.6	—	384
Admin. thyroidin	46.8	10.9	25.3	7.7	52.2	10.2	40.9	33.1	430
" "	32.1	4.7	21.4	2.8	45.0	7.2	42.4	—	424

The absence of marked changes in the ascorbic acid content of rat tissues does not, nonetheless, prove that the thyroid exerts no influence on ascorbic acid metabolism in these animals. Since rats are among the animals which synthesize ascorbic acid it seems possible that variations in its utilization are compensated by changes in its synthesis. In order to obtain an idea of the ability of tissues to utilize ascorbic acid a number of experiments was performed in which the rats were given subcutaneous injections of an aqueous solution of ascorbic acid calculated on 70 mg per 100 g body weight; the ascorbic acid content of tissues was determined 20 hrs later. Injection of ascorbic acid increased its content in the organs of normal and experimental rats. In this case, as in the experiments without administration of ascorbic acid, it was seen that the ascorbic acid content of liver, adrenals and testicles was lower in the hypothyroid rats (Table 5).

The same organs in hyperthyroid rats showed a higher concentration of ascorbic acid following injection of the latter than was the case in normal rats given the same amount.

DISCUSSION OF RESULTS

The results of investigation into the reduced glutathione content of liver in rats with experimental hypothyroidism induced by administration of a destructive dose of radioactive iodine or by 6-MTU indicate that the functional state of the thyroid plays an important role in glutathione metabolism. The glutathione content showed an increase whether the experimental hypothyroidism was produced by administration of radioactive iodine or by 6-MTU. The opposite effect was noted in hyperthyroidism. Fatterpaker [9] also notes diminution of glutathione in the liver and adrenals of rats given thyroxin which, in the author's view, is explained by impairment of its synthesis as the result of thyroxin-inhibited oxidative phosphorylation. The increase of glutathione in the liver of hypothyroid rats can, apparently, be explained by its decreased breakdown associated with diminution of oxidative processes. Considerable diminution of the latter is indicated by the data obtained by Watts [10] who observed decreased O_2 utilization in rats 8-12 days after administration of radioactive iodine.

Our results agree with the results obtained by Gros and Kirnberger [8] who observed a rise of blood glutathione in patients with hyperactivity of the thyroid treated with 6-MTU and its diminution upon administration of thyroxin. The authors note the inverse relation between the basal metabolic rate and blood glutathione content.

The data obtained by us give rise to the suggestion that the increase of glutathione may be one of the factors in impairment of carbohydrate function of the liver in animals and man subjected to administration of 6-MTU, as referred to in the literature [11].

The possibility of such a relation is determined by the fact that HS-glutathione is known to inactivate insulin. At the same time, administration of insulin to patients on 6-MTU exerts a beneficial effect on the carbohydrate and detoxicating liver functions [11].

Changes in the function of the thyroid have much less influence on the level of ascorbic acid in rat viscera. It was found in our investigations that only a slight decrease in free ascorbic acid content of liver and testicles occurred in animals given radioactive iodine or 6-MTU.

Similarly to other investigators, we failed to discover changes in the ascorbic acid levels of organs in hyperthyroid rats.

It must be mentioned that in this respect rats differ greatly from other animals, such as rabbits and guinea pigs. According to literature data, the latter show increased ascorbic acid levels on thyroidectomy and marked diminution of ascorbic acid on administration of excessive hormone.

According to Goldshtein et al. [7] decrease in free ascorbic acid in hyperthyroidism is caused by its passage into the iron-bound form.

Many authors have established that animals which synthesize or do not synthesize ascorbic acid react differently to the influence of one and the same factor. Thus, guinea pigs respond to administration of streptocide, medinal, and luminal by a decrease in ascorbic acid, while rats respond by an increase in the latter [13-15].

Slight changes in ascorbic acid level in hypo- and hyperthyroidism do not exclude participation of the thyroid in the metabolism of this compound. It would be more correct to postulate that rats, being animals capable of synthesizing ascorbic acid, react to changes in its utilization by change in the intensity of its synthesis.

Differences in ascorbic acid metabolism were observed in experiments with subcutaneous injection of the substance. In this case the viscera of hypothyroid rats (liver, testicles, adrenals) retained ascorbic acid to a lesser degree than the viscera of animals given thyroidin. This agrees with the data of Khvoinitskaia [6] who observed that the liver in hyperthyroid rats possessed greater capacity for reducing added dehydroascorbic acid than the liver of normal rats.

We could not detect any parallelism between the glutathione and ascorbic acid content which might have been expected on the basis of the protective action of glutathione against oxidation of ascorbic acid. On the contrary, changes in glutathione and ascorbic acid content were opposite in character, viz. in hypothyroidism the glutathione content increased while the ascorbic acid content decreased; in hyperthyroidism there was diminution of glutathione with unchanged content of ascorbic acid and greater retention of ascorbic acid upon its administration.

SUMMARY

Destruction of the thyroid by radioactive iodine or suppression of its function by 6-MTU is accompanied by increased concentration of reduced glutathione in the liver. The same animals show slight decrease in the ascorbic acid content of the liver and testicles. Its level remains unchanged in other viscera.

Ascorbic acid administered to rats is retained in the tissues of hypothyroid rats to a lesser extent than in animals given thyroidin.

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THE EFFECT OF X-IRRADIATION ON HYPOXANTHINE BIOSYNTHESIS AND ON GLYCINE METABOLISM IN PIGEON LIVER

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The investigation of purine biosynthesis is, apart from general biochemical interest, of considerable importance from the radiobiological point of view.

The inhibition of nucleic acid biosynthesis is one of the most characteristic metabolic effects caused by the action of ionizing radiation within the organism [1-3]. The actual point of lesion in the chain of reactions concerned in the biosynthesis of nucleic acids, most affected in radiation syndrome, is as yet unknown.

According to Harrington and Lavick [4], x-irradiation inhibits the neosynthesis of purine bases, without interfering with their incorporation into nucleic acids. Thus, irradiation of rats with 100 r inhibited by 50% the incorporation of formate-C^{14} into the desoxyribonucleic acid (DNA) thymine, while the incorporation of adenine-C^{14} into the adenine and guanine of DNA was not appreciably affected. The results of Harrington and Lavick [4] were basically confirmed by Passoneau and Trotter [5]. However, considerable inhibition of adenine-C^{14} incorporation into DNA, following x-irradiation, was claimed by other authors [6].

It was therefore of interest to investigate the effect of x-irradiation on purine biosynthesis under conditions where this process is not complicated by other reactions concerned in the biosynthesis of nucleic acids.

It is well known that intensive biosynthesis of a free purine – hypoxanthine – occurs in the pigeon liver, and therefore this system is a classical one for the study of the mechanism of purine biosynthesis.

The specificity of purine metabolism in the pigeon liver is, apparently, conditioned not by the peculiarity of the pathways for the purine ring synthesis, but by the complete absence of a turnover of the already synthesized base, hypoxanthine.

In this connection it seemed advantageous to utilize the pigeon liver in the investigation of the effect of ionizing radiation on the purine ring biosynthesis. It is known that one of the characteristic effects of x-irradiation on metabolism is a negative nitrogen balance [3]. The data on the effect of irradiation on hypoxanthine biosynthesis may therefore be of interest not only in connection with the study of nucleic acid metabolism in radiation syndrome, but also in the study of the effect of radiation on the final products of nitrogen metabolism in the organism, where the main portion of hypoxanthine is converted into uric acid.

EXPERIMENTAL

Methods

The source of x-rays used was a tube RUP, 180 kv, 15 ma, λ - 0.15 A. Filters: 0.5 mm Cu and 0.75 mm Al.

Irradiation intensity – 27 r/min. Total dose – 2000 r. The pigeons were killed by decapitation after a definite period (30 min to 12 days) bled, and the livers were removed and homogenized. One irradiated and one control pigeon was used in each experiment.

The main methods of investigation were isotopic tracing and paper chromatography. In most experiments, glycine-C^{14} was used as the starting material in hypoxanthine biosynthesis; formate-C^{14} was used in one experiment. Radioactivity on chromatograms was detected by autoradiography, and also with a sclaer of the "B" type with an end-window counter; the counting efficiency coefficient was 20%.

In order to detect the metabolic products of the hypoxanthine precursors, these substrates were incubated with the liver homogenates.

A typical incubation mixture is given below:

Liver homogenate	4 ml
Glycine-1-C^{14}	75 μC (1.1 mg) in 0.2 ml H_2O
$NaHCO_3$	20 mg in 1 ml H_2O

Liver homogenates were prepared by homogenizing the tissue with an equal volume of water in a glass homogenizer. Incubations were carried out for one hour at 37-39°. Reaction mixtures were de-proteinized by the addition of 3 ml of water-saturated n-butanol and subsequent standing in a thermostated bath (40-45°), with periodic shaking, for 1.5-2 hrs. The floculated proteins were filtered off. The filtrates were chromatographed, in the descending direction, on "fast" Leningrad paper. Strip length – 45-50 cm. The solvent mixture used was water-saturated n-butanol – 85% formic acid (90:10).

Chromatograms were dried, inspected in ultraviolet light and placed in x-ray casettes (usually for 20 days) for autoradiography. In the quantitative determination of hypoxanthine radioactivity the corresponding spot was cut out from the chromatogram, the paper segment thus obtained was pressed together with a new sheet of paper by means of two pieces of glass, and the chromatography repeated with water-saturated n-butanol as the mobile solvent and 3% ammonia in the bottom of the chamber.

This double treatment assured the purity of the hypoxanthine spot. (For details of the method see [7-9]).

RESULTS

The effect of irradiation on glycine-1-C^{14} metabolism and on hypoxanthine biosynthesis is illustrated qualitatively in typical photographs of autoradiograms (Fig. 1) (a total of 30 experiments were carried out).

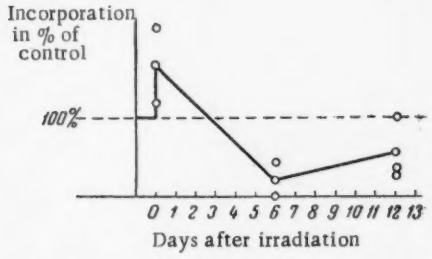

Fig. 2. The effect of total body irradiation (2000 r) on the intensity of glycine-1-C^{14} incorporation into hypoxanthine in pigeon liver homogenates.

It is seen from Fig. 1 that an initial activation of hypoxanthine took place immediately after irradiation and that this was followed later by a sharp depression. Incorporation of C^{14} from glycine-1-C^{14} into malic, succinic and fumaric acid was also inhibited. It was observed in the course of the experiments that the actual amount of preformed glycine in the liver was only slightly lowered in this period.

The qualitative results quoted above were also confirmed by quantitative analysis (Fig. 2). Both biosynthesis and actual content of hypoxanthine increased slightly in the livers immediately after irradiation with 2000 r, compared with controls, but fell considerably in the later stages.

The effect of irradiation on the enzymatic systems of hypoxanthine biosynthesis was not direct. The most important effect, namely severe inhibition of its activity, took place only several days after irradiation. It follows that the primary biochemical changes which took place during, or directly after, irradiation, caused only a gradual development of the significant and easily detectable changes of the enzymatic system. Experiments carried out in vitro showed that direct irradiation of the liver homogenate (without total body irradiation) had no effect on glycine metabolism even when doses up to 80,000 r were employed (Fig. 3).

The irradiation of homogenates in vitro was carried out in special plexiglass dishes. Homogenates were irradiated in layers 4-5 mm thick. Control homogenates were treated similarly, without irradiation. Irradiations were carried out with a short-focus x-ray tube over a period of 5-10 min, at room temperature.

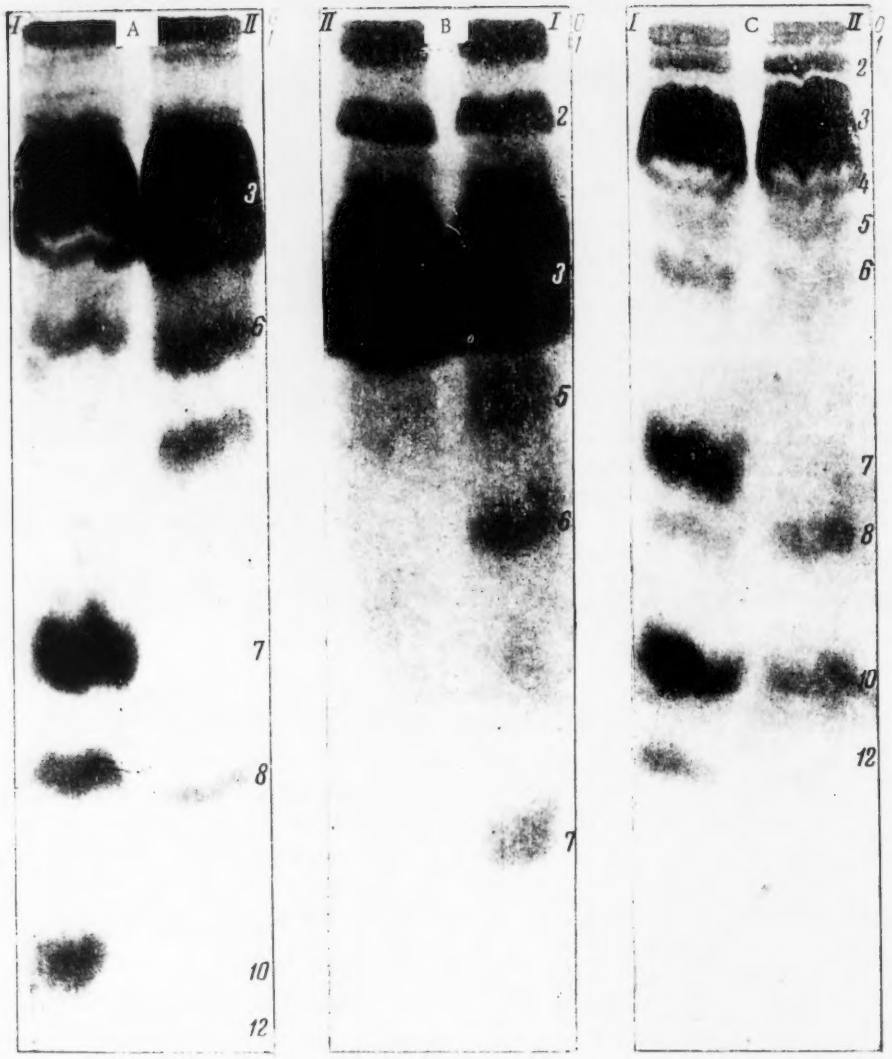

Fig. 1. Effect of total body irradiation (2000 r) on the metabolism of glycine-1-C^{14} in pigeon liver homogenates (autoradiograms). I) Nonirradiated control; II) after irradiation; A) immediately after irradiation; B) 6 days after irradiation; C) 12 days after irradiation; 0) origin of chromatograms; 1) nucleotide fraction; 2) unidentified fraction; 3) glycine-1-C^{14} residue; 4) inosine; 5) glutamic acid; 6) hypoxanthine fraction; 7) malic acid; 8) glycollic acid; 10) succinic acid; 12) fumaric acid. The lower fractions in chromatogram B are absent, since chromatograms were allowed to overrun.

Fig. 3. Glycine-1-C^{14} metabolism in normal and irradiated (80,000 r) pigeon liver homogenates. I) Nonirradiated control; II) irradiated sample; 0) origin on chromatograms; 1) nucleotide fraction; 2a and 2b) unidentified fractions; 3) glycine; 4) inosine; 5) glutamic acid; 6) hypoxanthine; 7) malic acid; 8) glycollic acid.

Fig. 4. Effect of total body irradiation (2000 r) on glycine-1-C^{14} metabolism in pigeon bone marrow homogenates. I) Nonirradiated control; II) 6 days after irradiation; 0) origin in chromatograms; 1 and 2) unidentified fraction, the metabolism of which is considerably disturbed by irradiation; 3) glycine-1-C^{14} residue.

Glycine-1-C^{14} metabolism in the pigeon bone marrow homogenate, immediately following 2000 r total body irradiation, did not differ from that of a normal pigeon. However, marked disturbances in the metabolism of glycine-1-C^{14} in this tissue were observed six days after irradiation (Fig. 4).

We have also shown in the above-mentioned experiments, that no incorporation of C^{14} from glycine-1-C^{14} into purine bases took place in bone marrow homogenates.

A possible reason for the intensification of hypoxanthine biosynthesis immediately after irradiation may be the accumulation of ribose-5-phosphate, resulting from nucleotide decomposition after irradiation. Such a decomposition of nucleotides after x-irradiation was reported by Hickman and Ashwell [10]. The strong activating

effect of ribose-5-phosphate on hypoxanthine biosynthesis is known from the literature, and was also confirmed in the present work.

The experiment with formate-C^{14} showed that the labile phase in hypoxanthine synthesis was connected with the inhibition of formate incorporation into a fraction which, in our previous communication [7-9], was referred to as "fraction 5"; the latter is a product of formate combination with folic acid, or with its derivative, and participates in purine biosynthesis. The position of this fraction may be seen in Fig. 1 (see Fig. 1, autoradiogram B, position 5).

It was also observed that the addition of glutamine, pyruvate and formate to the liver homogenate brought the incorporation of C^{14} from glycine-1-C^{14} into malic, succinic and fumaric acids closer to normal values. Hypoxanthine metabolism was also brought considerably closer to normal conditions. At the same time, the total available information points to the fact that, in purine biosynthesis, glutamine and pyruvate participate in the reactions of amination and carboxylation, respectively.

On this basis it is probable that the interference with hypoxanthine biosynthesis is connected with the inhibition of the processes of formylation, carboxylation and amination.

The most widely accepted scheme for hypoxanthine biosynthesis is that of Greenberg [11], according to which the synthesis takes place with the participation of ribose-5-phosphate and with the intermediate formation of glycinamide ribotide and carboxamide ribotide.

The scheme of Greenberg (with insignificant modification) is shown in Fig. 5, on which the most probable radiation disturbances of purine biosynthesis have been marked.

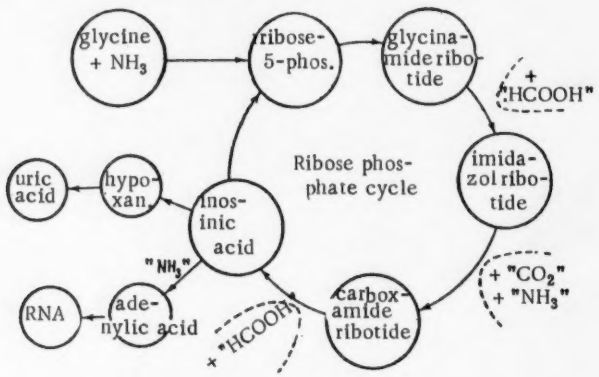

Fig. 5. A scheme for hypoxanthine biosynthesis interrelations with some radiation disturbance. ------) The most probable radiation disturbances in purine biosynthesis (formylation, carboxylation and amination reactions).

In analogy with the ribose phosphate ring, shown in Fig. 5, there is, apparently, in existence a similar ring involving desoxyribose phosphate, concerned with the synthesis of desoxyribonucleotides, which are the substrates for desoxyribonucleic acid biosynthesis.

According to literature, a certain optimal quantity of purine bases in the tissues is necessary for the support of the normal vital activity of the organism.

Thus, the introduction into a normal animal of excessive amounts of adenine causes the degeneration of the bone marrow [12]. On the other hand, an increased demand for adenine takes place some time after x-irradiation [13]. Therefore, the sharp decrease in the purine ring biosynthesis, observed in the present work several days after irradiation, may have a definite negative effect on the state of the organism.

It is known that the majority of mammals die on the 6th-7th day after a dose of 600-700 r total body irradiation. The data given above point to a considerably higher resistance of pigeons to irradiation, which may be due to the more intensive purine ring synthesis in the pigeon as compared with the mammals.

It is known that a large potential store of energy of any enzymatic system brings about an increased resistance of the given organism to factors which affect this enzyme [14].

The data quoted in this communication, together with published results, point to the fact that, under the action of ionizing radiation, simplified biochemical systems, such as homogenates, extracts and isolated enzymes, show a considerably higher radioresistance than the whole body.

The sharp distinction in response to ionizing radiation between such autolytic systems and the living organisms depends,apparently, in the first instance on the fact that the metabolic processes in the living body are infinitely more complex than in such simplified systems. This circumstance would bring about an intensification and accumulation of the damage in the whole organism, as compared with the simplified system. In the intact organism a primary lesion of any one biochemical process invariably affects the harmonious progress of many other biochemical processes, themselves possibly already damaged by some primary radiation disturbance.

SUMMARY

It was shown that in liver homogenates from irradiated (2000 r) pigeons,hypoxanthine synthesis, as judged from the incorporation of glycine-1-C^{14}, was slightly increased immediately following irradiation, and greatly decreased in the following day. C^{14} incorporation from glycine-1-C^{14} into malic, succinic and fumaric acids fell sharply immediately after irradiation and remained low thoughout.

Radiation damage of purine biosynthesis is largely due to the inhibition of the metabolism of substrates concerned in purine biosynthesis (formate, glutamine and organic acids).

The high resistance to radiation of the pigeon, as compared with the majority of mammals, has been demonstrated.

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STABILITY OF SOME N-GLUCOSIDES OF AMINO ACIDS AND PEPTIDES IN AQUEOUS SOLUTIONS

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The aim of the present work was to prepare the simplest models of carbohydrate-protein linkage and to study their properties. Since carbohydrate-containing proteins do not usually show free aldehyde groups, the glucosidic linkage may serve as one of the possible forms of attachment of carbohydrate to protein.

Formation of a glucosidic bond to involve various functional groups of amino acid residues in the protein can be visualized ($-NH_2$, $>NH$, $-COOH$, $-SH$). The N-glucosidic linkage is very probable in certain proteins. The following facts support this postulate. According to the Japanese chemists Inone and Onodera, they succeeded in isolating N-glucosides of amino acids and of peptides by enzyme and alkaline ($Ba(OH)_2$) hydrolysis of certain proteins; the N-glucosides they obtained were: N-glucoside of serine and N-(N-D-glucosido-L-seryl)-L-tyrosine from raw silk [1], N-glucoside of arginine and N-[N-(N-D-glucosidoarginyl)-L-triptophanyl]-L-tyrosine from crude casein and N-D-glucosido-L-serine from herring roe [2]. The work of these authors was available to us only in the form of brief abstracts on the basis of which it was impossible to exclude completely the possibility of secondary formation of N-glucosides in the process of working up and separation of hydrolyzates.

The properties of some carbohydrate-containing proteins resemble those of N-glucosides of amino acids in their lability. Thus, for example, in serum proteins part of the carbohydrates split off even on simple dialysis or acidification [3]. Grassmann suggests the possibility of N-glucosidic linkage of carbohydrates in collagen on the basis of experiments on reduction of collagen with $LiBH_4$ [4].

N-glucosides of amino acids possess definite biologic activity. Thus N-glucosylglycine stimulates the growth of Lactobacillus gayoni [5].

Formation of a N-glucosidic linkage can, evidently, cause increased stability of proteins to enzymatic hydrolysis. It is known that, as a consequence of protection of the free NH_2-group by glucose, the N-glucoside amide of glycylglycine is stable to amino- and polyaminopeptidase [6]. There are observations showing that the action of erepsin on dipeptides is inhibited by the addition of glucose [7]. Treatment of casein with acetaldehyde is followed by decreased extent of enzymatic hydrolysis of casein [8].

Formation of an unstable N-glucosidic bond apparently takes place on simple mixing and boiling down of solutions of protein and glucose as shown by the easy splitting off of part of the attached glucose. At the same time, more stable 1-amino-1-desoxy derivatives of fructose may also be formed as the result of Amadori rearrangement [9]. The reactions mentioned may play a certain stabilizing part in the method of blood plasma and serum drying adopted in the Soviet Union and carried out in the presence of glucose [10].

N-glucosides of amino acids are also of interest because compounds have been isolated from the body which are identical with substances obtained from N-glucosides of amino acids upon Amadori rearrangement. They stimulate the attachment of amino acids to proteins of certain tissues in rabbit [11]. If it is also taken into account that formation of N-glucosides most probably constitutes the first stage of the so-called "discoloration" in various food-processing procedures [12] it becomes obvious that N-glucosides can play a very varied part both in the life of a living body and in the technology of foodstuffs preparation.

The stability of N-glucosides was tested by means of polarimetric determination of the appearance of glucose and increase of its content in the solution.

Using this method it had already been established that the amide of glycylglycine glucoside was stable in aqueous medium, decomposed quantitatively in 0.1 N HCl within 3 hrs and was completely stable in 0.1 N NaOH. The amide of sarcosine glucoside is less stable both in the acid and alkaline media. At pH 4.5 (phosphate buffer) both glucosides are hydrolyzed within 24-48 hrs to a certain constant positive value of the specific rotation of the solutions [6]. Other workers found that a number of amino acid glucosides (as Na salts) were hydrolyzed slowly in aqueous solutions and considerably more quickly in acidified solutions [13, 14]. On the basis of these data an opinion has been formed that N-glucosides are most stable at pH 7-8 and that in aqueous solution an equilibrium dependent on concentration exists between the three reaction components involved in the formation of N-glucosides [14].

The works cited and some others show that the behavior of individual N-glucosides in solution has only received episodic study and that the range of pH over which these compounds are stable has not been established experimentally.

Keeping in view the possible attempts to isolate N-glucosides of amino acids and peptides from carbohydrate-containing proteins it was essential to determine this range of pH and to discover the conditions under which the components reach an equilibrium. This formed the aim of the present work. The precision of the polarimetric estimations was checked by parallel determinations of the free amino acid formed by titration according to Sorensen.

EXPERIMENTAL

N-glucosides were synthesized according to the Micheel and Klemer method [13] which was only modified in that the reaction mixtures were not kept in a thermostat but were immediately concentrated in vacuo at 35-37°. Care was taken to exclude moisture during isolation of the glucosides by their repeated solution in absolute methanol and precipitation with ether, especially at those times when the N-glucoside precipitates were not covered with solvent.

The following N-glucosides were synthesized.

Na salt of N-glucoside of glycine ($C_8H_{14}O_7NNa$):

Found %: C 36.71; H 5.84; N 5.60. Calculated %: C 37.07; H 5.44; N 5.40.

$[\alpha]_D^{16.5^\circ} = -8.1 \pm 0.5^\circ$ (water, c = 7.87) 12 min after solution. Value cited in literature: $[\alpha]_D^{18^\circ} = -8$ (water, c = 1) 10 min after solution.

Na salt of N-glucoside of diglycine ($C_{10}H_{17}O_8N_2Na$):

Found %: C 37.53; H 5.78; N 9.14. Calculated %: C 38.00; H 5.38; N 8.87.

$[\alpha]_D^{19^\circ} = -1.8 \pm 0.2^\circ$ (water, c = 7.3); literature value: $[\alpha]_D^{20^\circ} = 7.32$ (water). Such a deviation of specific rotation as cited in the literature and found by us is evidently explained by the fact that under the milder conditions of synthesis a preparation was obtained which was much less contaminated by glucose.

Na salt of glucoside of serine ($C_9H_{15}O_8NNa$):

Found %: C 36.70; H 5.33; N 5.21. Calculated %: C 37.5; H 5.25; N 4.86.

$[\alpha]_D^{20^\circ} = -14.3 \pm 0.6^\circ$ ($^2/_3$N Na_2SO_3) 12 min after solution. Literature value: $[\alpha]_D^{20^\circ} = -15^\circ$ (water, c = 1) 10 min after solution.

Divergence in the carbon content in the case of the serine N-glucoside is explained by the marked hygroscopic properties of the preparation which make its analysis difficult.

Reactions of the N-glucosides of Glycine, Serine and Diglycine

All the N-glucosides obtained give a positive Fehling reaction on heating, give violet coloration on ninhydrin-treated paper and when applied to paper as concentrated solutions give a bluish fluorescence in ultraviolet light. Under these conditions glycine does not fluoresce and glucose gives only a weak fluorescence.

On heating with 1.24 N solution of Na_2CO_3 for 5 min and addition of Ehrlich's reagent (n-dimethylaminobenzaldehyde + HCl) the solution of N-glucoside of glycine gives a rose-red coloration, the solution of N-glucoside of serine gives a similar color but in order to obtain a positive reaction it has to be heated with Na_2CO_3 for 15 min, while the solution of N-glucoside of diglycine remains practically colorless even after 15 minutes' heating.

The N-glucosides studied give a characteristic reaction in which they acquire a brown color on being heated dry to 105° for 5 min. The glycine glucoside gives the strongest coloration while the glucoside of serine gives a weaker one. The glucoside of the dipeptide gives only a barely perceptible yellowing.

Investigation of the stability of the sodium salts of N-glucosides was carried out according to the following scheme.

A weighed sample of the N-glucoside salt, equal to 0.15-0.20 g, was dissolved in 3 ml buffer with the appropriate pH (3N acetate buffer for pH around 5.5 and ammoniacal buffer of 3N NH_4Cl and 3N NH_4OH for pH above 7). The pH values were measured by means of a glass electrode potentiometer. The solution obtained was filtered quickly through a layer of paper pulp on a porous filter and placed into the polarimeter tube in which the rotation was determined at room temperature at definite intervals of time.

The extent of glucoside decomposition was calculated according to the following equations.

The observed rotation of the solution (α) is determined by the sum:

$$\alpha = [\alpha_1]_D C_1 l + [\alpha_2]_D C_2 l ,$$

where $[\alpha_1]_D$ and c_1 are specific rotation and concentration in g per 1 ml solution for the glucoside, respectively, and $[\alpha_2]_D$ and c_2 are the specific rotation and concentration for glucose; l is the length of the tube in dm.

If $\underline{x}$ is the amount of decomposed glucoside in 1 mg, then $c_2 = \frac{180 \cdot x}{M}$, where M is the molecular weight of glucoside, and $c_1 = c - x$ (where $\underline{c}$ is the initial concentration of glucoside in the solution).

This gives, considering c = 100%,

$$y = \frac{([\alpha]_D - [\alpha_1]_D)100}{[\alpha_2]_D \frac{180}{M} - [\alpha_1]_D}$$

where $\underline{y}$ is the amount of decomposed Na salt of N-glucoside in % and $[\alpha]_D$ is the specific rotation of solution with reference to the initial concentration of N-glucoside.

The value taken for glucose is $[\alpha_2]_D = 52.5°$, for N-glucoside of glycine $[\alpha_1]_D = -8°$, for glucoside of serine −15° and for the glucoside of glycine the average value of the first six determinations after solution at pH 9.9, viz. $[\alpha_1]_D = -3.8°$ (see Table 5).

Stability of the Na Salt of N-Glucoside of Glycine in Water

Experiments performed with solutions of various concentrations (Fig. 1) show that the Na salt of N-glucoside of glycine is hydrolyzed in aqueous solution to a certain state of equilibrium which under the given conditions is reached within 150-170 hrs. The more dilute the glucoside solution, the greater the decomposition into glycine and glucose.

The effect of the pH of the solution on the course of hydrolysis of the N-glucoside of glycine is presented in Fig. 2.

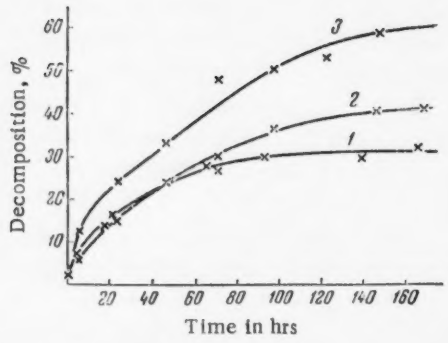

Fig. 1. Stability of N-glucoside of glycine solution in water depending on its concentration: 1) c = 0.0767 g/ml, pH at end of experiment 9.97; 2) c = 0.0243 g/ml, pH 1.5 hr after solution 9.5, at end of experiment 10.1; 3) c = 0.0081 g/ml.

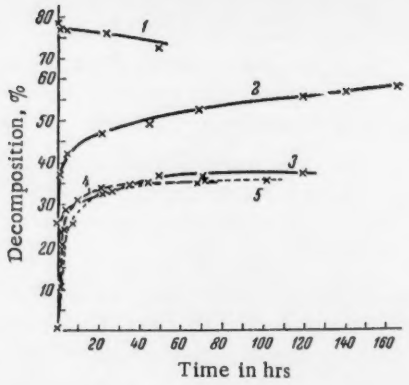

Fig. 2. Effect of the pH of solution on course of hydrolysis of the N-glucoside of glycine: 1) pH 5.4, c = = 0.0697 g/ml; 2) pH 7.7, c = 0.0609 g/ml; 3) pH 9.6, c = 0.0525 g/ml; 4) pH 10.0, c = 0.0557 g/ml; 5) pH 10.5, c = 0.0540 g/ml.

TABLE 1

Change in Optic Rotation of a Mixture of Glucose and Glycine in Solution at pH 9.9 (glucose 0.0368 g/ml calculated with respect to glucoside c = 0.0525 g/ml, temperature 20° ± 0.5, l = 2 dm)

Duration of expt. in hrs	α	$[\alpha]_D$, with respect to glucose
0.5	3.63±0.02	49.3±0.3
20	2.75±0.01	37.4±0.1
46.5	2.23±0.03	30.4±0.4
69	1.94±0.02	26.4±0.3
140	1.49±0.02	20.3±0.3
164.3	1.44±0.02	19.6±0.2
188.5	1.38±0.01	18.7±0.1
212.5	1.37±0.01	18.6±0.1

TABLE 2

Change in Optic Rotation of Glucose at pH 9.7 (c = = 0.0367 g/ml, l = 1.48 dm, temperature 19.5 ± 0.5°)

Duration of expt. in hrs	α	$[\alpha]_D$, with respect to glucose
71.75	2.42±0.02	44.6±0.4
93.3	2.35±0.01	43.3±0.2
118.0	2.30±0.01	42.4±0.2
140.75	2.23±0.01	41.0±0.2
165.25	2.12±0.02	39.0±0.4
189.0	2.10±0.02	38.7±0.4
238.5	1.97±0.02	36.3±0.4

An experiment was then staged in which weighed samples of glucose and glycine were taken in such a way as to obtain a solution corresponding to a solution of the Na salt of N-glucoside of glycine with c = 0.0525 g/ml, as in the experiment represented by curve 3 at pH 9.6 in Fig. 2.

Results of this experiment are given in Table 1.

An analogous experiment was performed with a solution of pure glucose; 0.0918 g glucose dissolved in 2.5 ml ammoniacal buffer, pH 9.7 (Table 2).

Comparison of the specific rotation of glucose in an alkaline medium (pH 9.7) with the specific rotation of a mixture of glucose and glycine (pH 9.9) shows that the specific rotation of the mixture after 10 days is approximately half that of pure glucose which indicates that glucoside is formed in the case of the mixture of the two components.

Stability of the Na Salt of the N-Glucoside of Serine at Different pH of Solution

The data obtained in the appropriate experiments are presented graphically in Fig. 3.

The dependence of the state of equilibrium between the mixture of glucoside and glucose on the pH of solution was confirmed by the following experiment.

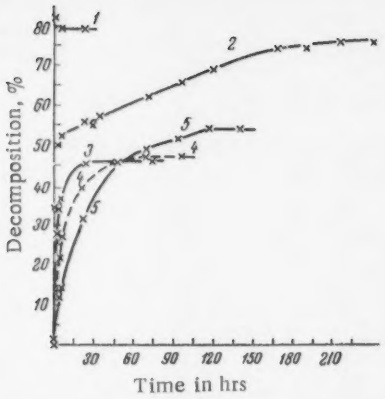

Fig. 3. Stability of the Na salt of the N-glucoside of serine at various pH values of the solution: 1) c = = 0.0647 g/ml, pH 5.6; 2) c = 0.0527 g/ml, pH 7.8; 3) c = 0.0827 g/ml, pH 9.7; 4) c = 0.061 g/ml, pH 10.6; 5) solution in $^2/_3$ N Na_2SO_3; c = 0.0483 g/ml, pH 9.8.

TABLE 3

Change in the State of Equilibrium in Solution of the N-Glucoside of Serine on Altering the pH (c = 0.0507 g/ml, l = 1.48 dm, temperature 19.5± 0.5°)

Duration of expt.	pH of soln	α	$[\alpha]_D$ with respect to glucoside	Decomp. of glucoside, %
10 min	5.5	1.78±0.02	23.8±0.3	81.1
1 hr	5.5	1.72±0.01	23.0±0.1	79.3
18.5 hrs	5.5	1.71±0.02	22.8±0.3	79.0
20.25 "	8	1.57±0.01	20.9±0.1	75.0
47.5 "	8	1.57±0.02	20.9±0.3	75.0
90 "	8	1.56±0.02	20.8±0.3	74.8

A solution of the N-glucoside of serine (c = = 0.0507 g/ml) was maintained at a pH of 5.5 for 18 hrs and, as can be seen from Table 3, an equilibrium with 81% decomposed glucoside was reached within 10 min. The state of equilibrium subsequently remained almost unaltered. The solution was then neutralized with solid $NaHCO_3$ to pH 8 and again placed in the polarimeter tube. The equilibrium shifted towards the glucoside.

If the amount of Na salt of the N-glucoside of serine decomposed after making the solution alkaline in this experiment is compared with its amount in the preceding experiment (Fig. 3, curve 2) when equilibrium had been reached, it is seen that they are practically identical. This indicates that in the experiment with initial pH 5.5 a certain amount of glucose and serine recombined on addition of alkali, creating a state of equilibrium characteristic for pH ~ 8.

Checking of Polarimetric Measurements by Formol Titration of Amino Acid Liberated on Hydrolysis

In order to ascertain the precision of the polarimetric measurement of degree of glucoside hydrolysis one of the experiments was carried out with parallel determination of the amount of free amino acid by the formol titration method. This method proved to be applicable to alkaline solutions of glucosides provided the addition of formaldehyde followed immediately upon neutralization of the solution to pH 7.

Comparison of the results obtained by the two methods is given in Table 4.

TABLE 4

Determination of the Amount of Decomposed N-Glucoside of Serine by Polarimetry and Formol Titration (c = 0.0095 g/ml, in water, temperature 20 ± 0.5°)

Duration in hrs	Polarimetric determination			Formol titration		
	α	$[\alpha]_D$ with respect to glucoside	% decomp. of glucoside	Duration of expt. in hrs	0.02 NaOH in ml	% decomp. of glucoside
3	0.04±0.02	3.26±1.63	38.2±3,5	1.6	0.57	35.2
4	0,06±0.02	4,9 ±1.63	41.6	—	—	—
24	0,19±0.02	15.5 ±1.63	63.7±3.4	22.6	1.04	64.2
45.3	0,25±0.02	20.4 ±1.63	74.0±3.0	45.15	1.28	79.9

It follows from Table 4 that the amount of decomposed N-glucoside of serine calculated from polarimetric data corresponds fairly well to the data given by formol titration.

Stability of the N-Glucoside of Diglycine at Different pH Values of Solution

Data obtained in the appropriate experiment are presented graphically in Fig. 4.

Absence of decomposition at pH 9.9 during the first 48 hrs of the experiment is shown in Table 5.

TABLE 5

Decomposition of the N-glucoside of Diglycine at pH 9.9 (c = 0.0587 g/ml, l = 1 dm, temperature 19 ± 0.5°)

Duration of expt.	α	$[\alpha]_D$ with respect to glucoside	Decomp. of glucoside in %
10 min	−0.22±0.01	−3.7±0.2	0
70 "	−0.23±0.01	−3.9±0.2	0
2 hrs	−0.19±0.03	−3.2±0.5	0
2.75 "	−0.23±0.02	−3.9±0.3	0
20 "	−0.27±0.03	−4.6±0.5	0
43 "	−0.19±0.02	−3.2±0.3	0
67.5 "	−0.07±0.01	−1.2±0.2	7.5
91 "	0.00±0.02	0±0.3	11.3
140 5 "	0.11±0.02	1.9±0.3	16.9
163.1 "	0.18±0.02	3.1±0.3	20.5
192.3 "	0.21±0.02	3.6±0.3	22.0
211 "	0.24±0.03	4.1±0.5	23.0
235 "	0.25±0.02	4.3±0.3	24.0

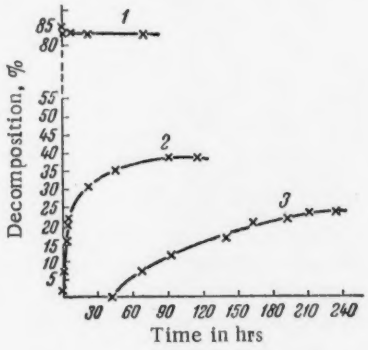

Fig. 4. Stability of the N-glucoside of diglycine at various pH of solution: 1) pH 5.6, c = 0.0677 g/ml; 2) pH 7.7, c = 0.060 g/ml; 3) pH 9.9, c = 0.0587 g/ml.

DISCUSSION OF RESULTS

As already indicated, the aim of the present work was to discover the range of pH over which the N-glucosides of amino acids were relatively stable in solution.

The glucosides studied did not show complete stability at any pH value of the solution. As can be seen from Figs. 2-5, the Na salts of the N-glucosides of glycine, serine and diglycine are hydrolyzed in buffer solutions of pH from 5.5 to 10.5 until a certain equilibrium is reached. The rate at which equilibrium is reached is different for each glucoside and for each pH; so is the point of the equilibrium itself. That a certain equilibrium is in fact reached is indicated by the change in rotation of a mixture of free glycine and glucose. After a period of 10 days the specific rotation of a solution of the mixture showed a drop which was double that observed in the case of a solution of pure glucose at the same pH and in the same buffer, which indicates the formation of glucoside. This is also supported by the following fact: when a solution of the N-glucoside, in a state of equilibrium at pH 5.5, was made alkaline (pH ~ 8) a state of equilibrium was reached which was characteristic for equilibrium at pH ~ 8.

Figure 1 shows that when the concentration of the N-glucosides in water increases the equilibrium is shifted toward the formation of glucoside, i. e., the stability of the glucoside to hydrolysis is increased. One of the reasons for this may be the association of glucoside molecules by way of hydrogen bonds when the concentration of the solution is raised.

Such behavior of N-glucosides should have been expected on the basis of the work carried out by Micheel and Klemer who found that formation of N-glucosides of amino acids from a mixture of amino acid, glucose and $NaHCO_3$ practically did not take place at high dilutions and only occurred on evaporating down the solutions [13].

All three glucosides under investigation are hydrolyzed almost instantaneously to the extent of 70-80% in weakly acid media (acetate buffer, pH 5.5), i. e., at pH 5.5 they are practically unstable. On raising the pH from 5.5 to 9.7-9.9 their stability increases but remains almost unaltered on further increase in pH to 10.5 (Figs. 2, 3, 4).

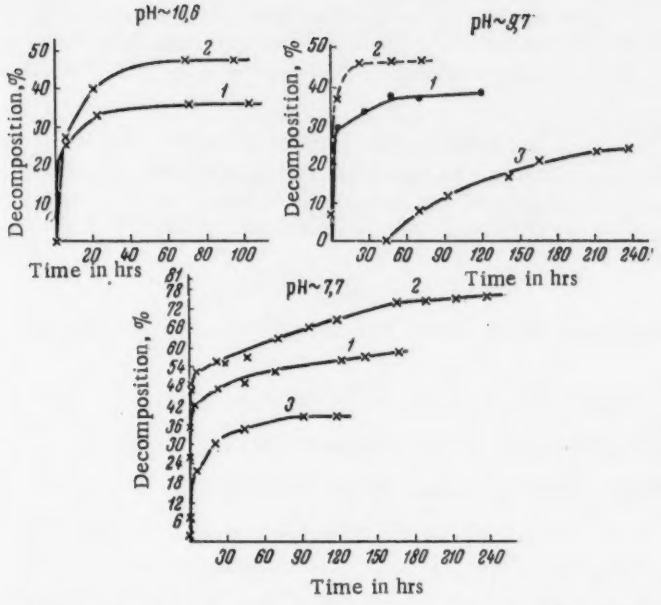

Fig. 5. Decomposition of glucosides at pH 10.5, 9.7 and 7.7: 1) N-glucoside of glycine; 2) N-glucoside of serine; 3) N-glucoside of diglycine.

A similar type of change under the influence of pH of the medium has been observed by von Euler and Zeile [15] in the case of the ethyl ester of the N-glucoside of glycine and by Kuzin and Poliakova [16] in the case of the Ca salt of the same glucoside.

Figure 5 shows that the diglycine glucoside decomposes to the least extent, followed by the glycine glucoside and by the glucoside of serine which decomposes to the greatest extent.

The stability of the N-glucoside of diglycine is so much higher than that of the amino acid glucosides that at pH ~ 9.9 it remains practically undecomposed over a period of 2 days (Fig. 5, curve 3 and Table 5). A repeat experiment at pH 10.5 gave similar results; decomposition of the glucoside of diglycine only became noticeable after being in solution for 3 days.

The nature of the buffer is also known to affect stability in solution. As can be seen from the slope of the curves in Figs. 1 and 2, the rate of hydrolysis in water without the addition of buffer is lower than in ammoniacal buffer, despite the similar value of pH ~ 9.6 (curve 1, Fig. 1 and curve 3, Fig. 2).

The glucosides obtained in the present work showed marked fluorescence in ultraviolet light.

According to the work of Friedmann and Kline [17] fluorescent compounds are formed upon interaction of amino acids with carbohydrates (at pH 5.2 and 121°); the fluorescence increaseson addition of $NaHSO_3$ to the reaction mixture. We put forward the suggestion that this may occur as the result of increasing the concentration of glucoside under these conditions. In such case it would be logical to expect increased stability of glucosides in $NaHSO_3$ or Na_2SO_3 solution. We carried out such an experiment in the presence of sodium sulfite (curve 5, Fig. 3) and obtained some increase in stability during the first 50 hrs. However, during the next 80 hrs hydrolysis proceeded even somewhat further than without addition of sulfite.

SUMMARY

The Na salts of the N-glucosides of glycine, serine and diglycine are hydrolyzed to a certain equilibrium state in buffer solutions of pH ~ 5.5, ~ 7.7, ~ 9.8, and ~ 10.5 at ~ 20°. The rate at which the equilibrium is

reached as well as the composition obtaining at the state of equilibrium differ for each salt and for each pH value of the solution. The equilibrium shifts toward the glucoside when the concentration of N-glucoside is increased.

The N-glucosides studied are almost instantaneously hydrolyzed to the extent of 70-80% at pH 5.5 (acetate buffer), i. e., under these conditions they are practically unstable. The stability of the N-glucosides under investigation grows on changing the pH from 5.5 to 9.7 and then shows little increase on raising the pH further to 10.5. It has been found that the zone of relative stability for N-glucosides is not pH 7-8 as had been thought [14] but a more alkaline one.

The N-glucoside of serine is less stable than that of glycine. The N-glucoside of dipeptide (diglycine) is considerably more stable than the amino acid glucosides. Its higher stability is expressed particularly vividly in that at pH 10 it is practically unhydrolyzed over a period of 2-3 days, whereas decomposition of the amino acid glucosides under these conditions begins within the first minutes of solution.

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THE MECHANISM OF POTASSIUM BROMATE ACTION AS AN IMPROVER IN BREAD BAKING

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One of the most widely used improvers in bread baking is potassium bromate, a compound in use more than 40 years ago. There is as yet, however, no single opinion as to the nature of the improving effect of $KBrO_3$ on wheat flour.

The broadest and earliest hypothesis which to some degree explained the specific action of $KBrO_3$ was that of Jørgensen [1]. Some experimental trials he made showed that $KBrO_3$ can inhibit the action of proteolytic enzymes in the flour which bring about disintegration of protein substances during dough handling. Jorgensen considered that as a result of the partial slowing of proteolysis due to addition of $KBrO_3$, the gluten protein retains its inherent properties of expansibility and elasticity to the necessary degree, as a result of which there is observed an increase in the gas-holding capacity of the flour.

Later this hypothesis was criticized and the opinion which was expressed placed in doubt the very significance of proteolytic enzymes in the biochemistry of bread baking. However, it was shown that flour proteinase can act energetically on the gluten when activated by glutathione, cysteine, ascorbic acid and other natural proteolytic activators [2]. It can also be assumed that substances with an oxidative character, as potassium bromate and iodate, can react with the indicated activators, in this way blocking their activating effect on proteinases.

Nor should the possibility be excluded of the direct effect of oxidative substances of the type indicated on the gluten protein, thus altering its enzymatic attackability [3], as a result apparently of changes in intra- or intermolecular sulfur bonds.

Thus, in establishing the true mechanism of improving action of oxidizers on flour, it can be assumed that the oxiders interact with the most vulnerable compounds (most inclined to participate in appropriate reactions) and also with the individual groups comprising the given compounds. Such substances in the dough are primarily free thiol groups – chiefly glutathione in the flour and yeast – and protein substances formed like gluten and also a number of water soluble fractions, among them various enzyme systems.

Quite recently the hypothesis was expressed [4] that the mechanism of oxidative improvers can play a significant role also in lipids.

From the foregoing, it is evident that the correct interpretation of the indicated mechanism of $KBrO_3$ action as an improver in bread baking is a difficult task.

It seemed to us that in deciding these questions, an investigation of the rate of change of $KBrO_3$ when added to flour, dough, yeast, gluten and directly to glutathione preparations, could be of assistance, and this was the purpose of the present study.

EXPERIMENTAL

Materials and Methods

The material used in the studies was commercial wheat flour of various varieties and quality, to which in a series of tests was added a batch of defatted wheat germ to increase the glutathione content. Tests were also

set up with yeast dough during fermentation. In addition to the usual pressed yeast, dry yeast from a plant drier was used.

Determination of the residual $KBrO_3$ was conducted by the Howe [5] method, based on oxidation of added iodide to iodine in the presence of $KBrO_3$ with subsequent titration of iodine formed by thiosulfate. Substances which interfered with the determination were first removed by hydrated zinc oxide at pH 6.85-6.90. The method is simple and very sensitive.

The tests were carried out as follows. To 10 g flour or dough with the addition of the required quantity of $KBrO_3$ added with constant mixing or grinding with sand in a mortar, 200 ml of distilled water was added in 20-40 ml portions. The homogenized suspension was left for 10-15 min, after which was added 25 ml 0.18 N $ZnSO_4 \cdot 5H_2O$ and 25 ml 0.18 N NaOH, the mixture stirred and filtered or centrifuged after 5-10 min. 50 ml of clear solution was acidified with 10 ml 10% H_2SO_4, 3 ml freshly-prepared 3% KI solution and 1 ml 1% starch added, and titrated with 0.001 N thiosulfate to decoloration. A control determination was conducted by titrating 0.001 N $KBrO_3$ with thiosulfate under the same conditions. Each 50 ml filtrate or centrifugate corresponds to the content of $KBrO_3$ in 2 g flour or dough.

Control tests were also set up with flour, but without addition of $KBrO_3$, in which case no formation of iodine was detected.

RESULTS

Determination of Residual $KBrO_3$ in Flour Suspensions

To a 10 g sample of first-quality flour was added the above amount of water containing 0.3 mg $KBrO_3$. After a 3-min agitation, the mixture was held with toluene at 27° for various periods, after which the determinations indicated were carried out.

In all the tables there are given the direct data as to titration in ml $Na_2S_2O_3$ per 2 g original material, without any conversion.

From Table 1 it can be seen that in the course of a 6-hr test there was observed a regular but slight loss of $KBrO_3$, similar in both samples of number 1 quality flour.

TABLE 1

Determination of Residual $KBrO_3$ in Flour Suspensions in ml 0.001 N $Na_2S_2O_3$

Sample	Time in hrs				Loss
	0	2	5	6	
First quality flour	1.90	1.84	1.76	1.65	0.25
First quality flour (another sample)	1.86	1.82	1.78	1.62	0.24

Determination of Residual $KBrO_3$ in Nonyeast Dough

Samples of flour of varying quality and flour with different amounts of added defatted wheat germ were used in these tests. The dough was mixed under water containing an amount of $KBrO_3$ corresponding to 0.003%

TABLE 2

Determination of Residual $KBrO_3$ in Nonyeast Dough in ml 0.001 N $Na_2S_2O_3$

Sample	Time in hrs				Loss
	0	2	4	6	
Dough from first quality flour	1.90	1.75	1.50	—	0.40
The same (another sample)	1.88	1.62	1.36	—	0.58
Dough from very fine wheat flour	1.90	1.75	1.60	—	0.30
The same + 5% germ	1.82	—	0.90	—	0.92
The same + 10% germ	1.80	—	0.68	—	1.12
Dough, first quality flour with low gluten	1.88	1.68	1.47	1.20	0.68
The same + 5% germ	1.84	1.64	1.44	0.89	0.95
The same + 10% germ	1.82	1.42	1.05	0.65	1.17

of the weight of the flour. The dough proofing was conducted at 27° and 85% relative humidity for various times.

From the data in Table 2 it follows that with an increase in dough proof time, the amount of residual $KBrO_3$ gradually decreases in all cases. But the reaction rate of $KBrO_3$ depends on the quantity of iodine-reducing substances in the samples of flour studied. Thus, with an increase in germ, as a carrier of glutathione, there was a corresponding decrease in the residual $KBrO_3$ in the dough.

Determination of Residual $KBrO_3$ in Yeast Dough

In these tests the dough was mixed with 3% pressed yeast and also with various quantities of dry yeast. Fermentation was conducted at 32°.

The results shown in Table 3 indicate that the dough fermented with pressed yeast reacts with $KBrO_3$ in almost the same way as nonyeast dough, but with fermentation with dry yeast, $KBrO_3$ decomposition began sooner, proceeded more intensely, and depended upon the quantity of added yeast.

TABLE 3

Determination of Residual $KBrO_3$ in Yeast Dough in ml 0.001 N $Na_2S_2O_3$

Sample	Time in hrs				Loss
	0	2	4	6	
Dough from first quality flour, pressed yeast	1.89	1.70	1.56	1.42	0.47
Dough from low-gluten flour with pressed yeast	1.78	1.58	1.30	1.10	0.68
Dough from first quality flour with 1% dry yeast	1.88	—	1.30	—	0.58
The same with 2% dry yeast	1.86	—	0.65	—	1.21
The same with 2.8% dry yeast	1.82	0.96	0.48	—	1.34

Differences in the rate of the $KBrO_3$ decomposition in the dough during its fermentation with pressed and dry yeast can be explained on the basis of the nature of the penetrability of the yeast cells. Fresh pressed yeast with a relatively low quantity of dead cells releases practically no glutathione into the surrounding medium and firmly holds it in the cells. The quantity of released glutathione markedly increases in proportion to the number of dead cells and the onset of autolysis in the pressed yeast [6]. Dry yeast, on the other hand, releases glutathione very easily as a result of changes in permeability which come about as a result of drying [7].

TABLE 4

Determination of Residual $KBrO_3$ in Yeast Suspensions in ml 0.001 N $Na_2S_2O_3$

Sample	Time in hrs				Loss
	0	2	4	6	
Pressed yeast, 3%	1.90	1.90	1.88	1.88	0.02
Dry yeast, 2%	1.88	1.38	0.83	0	1.88

For further verification of these facts, special tests were set up with one yeast, both pressed and dry, in which the $KBrO_3$ was added to a suspension of the yeast which was in a condition of continual fermentation at 32° – in the presence of sugar.

From the data in Table 4 it can be seen that in the tests with pressed yeast in the course of a 6-hr fermentation practically no loss was noted in the amount added of $KBrO_3$. However, with dried yeast under similar conditions, the results were entirely different, inasmuch as in the same fermentation period there was complete decomposition of the $KBrO_3$.

The more intense decomposition of $KBrO_3$ in the dough with the use of dry yeast in practical bread baking requires, in our opinion, a corresponding increase in the level of $KBrO_3$ for the optimum range necessary to insure obtaining of high quality bread.

Tests with addition of $KBrO_3$ to acetate solutions of gluten of various qualities, which were then held up to 6 hrs at 32 and 50°, indicated that in the first instance there was no noticeable loss of $KBrO_3$ and only at 50° was complete loss observed.

Determination of Residual $KBrO_3$ and Glutathione When Present Simultaneously

Finally, tests were set up to determine interaction of glutathione with $KBrO_3$. Previously, Ziegler [8] went into the question of the dependence of the rate of oxidation of glutathione on pH, temperature and $KBrO_3$ concentration.

TABLE 5

Determination of Residual $KBrO_3$ and Glutathione When Present Simultaneously

Deter. residual $KBrO_3$ and glutathione	Time in hrs		Residual $KBrO_3$	
	ml 0.001 N $Na_2S_2O_3$	in %	in ml 0.001 N I_2	in %
0	1.90	100	0.95	100
2	1.75	91	0.75	78
4	1.62	85	0.60	63
6	1.45	76	0.55	57

To a glutathione solution in pH 4.95 acetate buffer $KBrO_3$ solution was added and the mixture held at 30° for various periods, after which there was determined both the residual $KBrO_3$ by the Howe method and the unreacted glutathione by titration in acid medium with 0.001 N iodine solution.

The results (Table 5) showed that in the course of a 6-hr test there was a steady decrease in the quantity of unreacted $KBrO_3$, and simultaneously a drop in the glutathione content not entering the oxidation reaction.

The reaction between the two substances can be hypothetically expressed by the following scheme:

Results of investigation of changes in KIO_3 labeled with radioactive iodine when added to flour for bread baking confirm this schematic reaction, since it was shown that almost all the labeled iodine appeared in the form of KI [9]. Thus, in cases of interaction of $KBrO_3$ with glutathione or with other thiol compounds, it is wholly natural to obtain similar results, that is, a reaction of reduction of $KBrO_3$ to KBr with concurrent oxidation of SH- to S—S-groups.

These final tests, just as the preceding ones, indicate that $KBrO_3$ when added to flour, dough and yeast, evidently reacts primarily with iodine-reducing substances present to some extent in the indicated materials. However, it seems to us premature to exclude completely the direct reaction of $KBrO_3$ with gluten proteins, despite the obtaining of inconclusive results. This question requires further study, and possibly application of more sensitive methods and changes in the test conditions.

SUMMARY

$KBrO_3$ when added to an aqueous suspension of flour or dough without or with yeast undergoes a gradual but noticeable decrease in concentration according to the length of the experiment. In the dough from flour with a greater content of iodine reducing substance, particularly upon addition of a yeast enriched with glutathione, the loss of $KBrO_3$ proceeded at a greater rate as compared with the dough from flour with a lesser content of the above substances. A pronounced difference was noted in the interaction of pressed and dried yeasts with $KBrO_3$. During a 6-hr fermentation period practically no loss of $KBrO_3$ was noted in experiments with pressed yeasts, whereas with dried yeasts it completely disappeared. In model experiments the interaction of $KBrO_3$ with glutathione has been demonstrated.

Hence, it may be concluded that the "improving" effect of $KBrO_3$ is a function of its oxidizing action which is presumably directed mostly toward substances with the properties of thiols.

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CHROMATOGRAPHIC SEPARATION OF 5'-PHOSPHATE ESTERS OF ADENOSINE AND INOSINE BY ION EXCHANGE RESINS

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Cohn and Carter [1] developed a method for separation of adenosine 5'-phosphates by ion exchange chromatography. In their tests a mixture of a few milligrams of adenosine phosphates were adsorbed at pH 8-9 on a column of the anion exchanger Dowex-1. The quantitative separation of the components of the mixture was attained by subsequent elution of the nucleotides in 0.003 M HCl (AMP),* 0.02 M Na Cl and 0.01 M HCl (ADP) and 0.2 M NaCl and 0.01 M HCl (ATP).

Reference is made to this principle for the preparative separation of AMP on a column of Dowex-1 with a 33 cm^2 cross section [2]. In subsequent work the separation of adenosine phosphates by the Cohn and Carter method was used mainly for analytical purposes [3, 4].

Chromatographic separation of inosine phosphates on the anion exchanger Dowex-2 was accomplished by Deutsch and Nilsson [4].

The present paper describes a method of chromatographic separation of adenosine and inosine phosphates for analytical and preparative purposes with domestic anion exchange resins.

EXPERIMENTAL

Resin. In most of the tests EDE-10-P and AV-16 were used.

The first of these [6] is a condensation product of polyethylenepolyamine and epichlorhydrin and contains as the functional groups chiefly secondary and tertiary basic aliphatic series, and also a certain number of quaternary ammonium bases. It is produced by the plastic factory in Leningrad (Ministry of Chemical Industry, USSR). The second is also an anion exchanger of the condensation type (as distinct from the ionite of the copolymer type, to which particularly belong the American anion exchanger resins Dowex-1 and Dowex-2); it contains mainly the same functional groups as the first. It is produced by the experimental plant in the science investigative and planning Institute of Plastics of the Ministry of Chemical Industry, USSR, Moscow.

The anion exchangers EDE-10, AN-2F, AN-9, and N-0 were also studied.

Grinding of most of the resin was carried out by a hand mill (used in grinding coffee beans). In working with especially hard resin (EDE-10), a porcelain ball mill was used. The N-0 resin was ground in a mortar.

In each case the resin before screening was dried on sheets of filter paper at room temperature for a day.

Screening was carried both on metal screens Nos. 30, 40 and 100 (75, 100 and 250 mesh, respectively), and on cloth sieves (120-130 mesh) used in the milling industry.

* Abbreviations: AMP – adenosinemonophosphate, ADP – adenosinediphosphate, ATP – adenosinetriphosphate, IMP – inosinemonophosphate, IDP – inosinediphosphate, ITP – inosinetriphosphate.

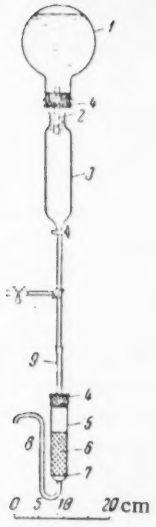

Fig. 1. Column for preparative separation of phosphate esters of adenosine and inosine by anion exchange resins: 1) 1000 ml flask; 2) 0.8 mm glass tube with side opening 5 × × 10 mm; 3) 250-ml separatory funnel; 4) rubber stopped; 5) glass tubing 16 cm long and 2.2 cm internal diameter; 6) resin layer 8.5 × 2.2 cm; 7) grid of sintered glass; 8) capillary manometer (internal diameter 2 mm); 9) glass tubing 4-5 mm diameter.

That fraction of particles which passed through the sieve with the smallest openings was suspended in water (0.5 liter per 15-20 g resin) and after 7-10 decantations the coarse fractions, settling in 1-3 min, as well as the finest particles.(those which did not settle in 15 min after suspension of the resin in water)were discarded.

The resin thus obtained, quite homogeneous in particle size, was used for transformation into the chloride form by suspension in 3-4 volumes M HCl and being allowed to stand for 15-20 min. After decantation the resin was carefully mixed with a small volume of M HCl until a thick homogeneous paste was obtained which was transferred to a glass column in a quantity sufficient for formation of a layer of the necessary height (the remaining resin was washed with several volumes of water and stored in wet form). The column was left overnight in strictly vertical position. By such means there was usually obtained an even filling of the column throughout; the surface of the layer of resin was strictly horizontal.

The column, somewhat simplified according to the suggestion of A. A. Baev (Institute of Biochemistry, A. N. Bakh Academy of Sciences USSR) by comparison with ones described in the literature [7], had an internal diameter of 2.2 cm and a height of resin layer 8.5-9 cm (for preparative separation) or an internal diameter of 0.8 cm with a height of resin layer of 3-4 cm (for analytical separation).

The column for preparative separation is represented in Fig. 1.

The resin layer confined above and below by discs of compact paper is placed on the sintered glass grid (pore diameter – 1 mm). A decrease in the dead space at the lower end of the column in which mixing of the various fractions might have occurred was achieved by: 1) use of the capillary manometer (8, Fig. 1) with an internal diameter of 2 mm, and 2) a sharp turn in tube 5 with a 2.2 cm diameter with a thin outlet connected to the capillary manometer by rubber tubing with a 2.5 mm internal diameter. It does not however follow that excessive reduction of the dimensions of the funnel-shaped neck in the lower portion of the column and an increase in the diameter of the column avoid formation of "pockets" at the lower cutoff of the column with a slowing of the flow of solvent. The space above the resin surface is entirely convenient for filling with solvent according to our observations. A constant level of solvent in the separatory funnel(3)is maintained on the principle of the "Mariott vessel" (1 and 2, Fig. 1).

The rate of flow of the solvent through the column is ordinarily regulated by changing the height of the water column above the resin layer. The optimum rate is 1 ml of solvent per min per cm^2 of cross section.

After introduction of the resin into the column, 2-3 volumes of M HCl is put through and it is then washed with 1.5-2.1 of water until the solution passing through gives a weakly acid reaction (pH 4.5-5).

Analytical Separation of Adenosine 5'-Phosphate Esters

The material investigated was a commercial preparation of free adenosinetriphosphate which had been stored at room temperature for 2.5-3 years (Ivanov meat combine, Ministry of Industrial Supplies USSR) containing a mixture of AMP, ADP and ATP.

Adsorption of nucleotides in the upper portion of the resin column is almost complete when the investigated material is put through the column in water solution, at the rate of 1 ml/min per cm^2 cross section, the water solution first being adjusted to pH 8.5 with ammonia. (The concentration of the ATP solution not exceed-

ing 500 mg%). In this process there usually appears a white ring in the upper portion of the resin layer, clearly evident against the yellow-brown background of the resin. In washing with water (with a volume equal to the volume of the solution being investigated) the position of this ring does not change. Upon elution it gradually disappears.

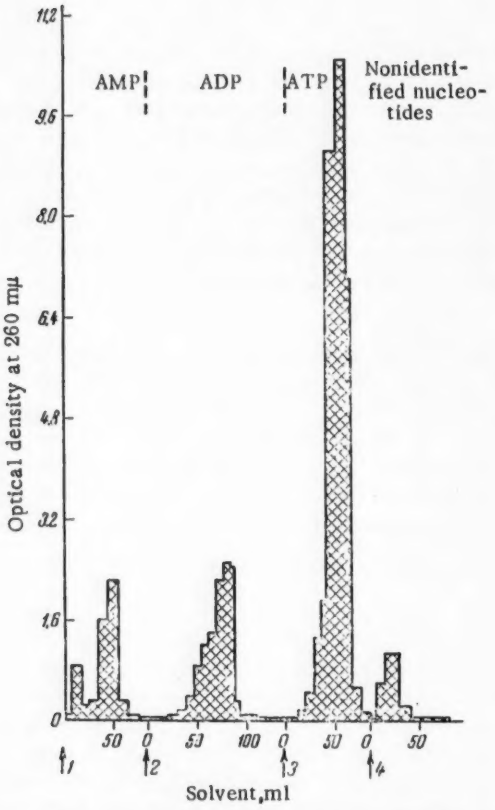

Fig. 2. Separation of analytical quantities of adenosine 5'-phosphate esters: resin AV-16, 250 mesh, column 3 × 0.9 cm (internal diameter), Cl, flow rate 0.2 ml/min per cm^2, material investigated – 20 mg commercial preparation of ATP (production date – March 1954). 1) Start of elution with 0.1 M NaCl in 0.1 M HCl; 2) start of elution with 0.2 M NaCl in 0.1 M HCl; 3) start of elution with 0.2 M NaCl in 0.2 m HCl; 4) start of elution with M HCl. Total yield of eluted nucleotides – 90%.

The optimum load for a column of the above-indicated dimensions is 20-30 mg of a nucleotide mixture.

Nucleotide extraction (elution) is conducted by the following solvents: 1) 0.1 M NaCl in 0.01 M HCl (AMP); 2) 0.2 M NaCl in 0.1 M HCl (ADP); 3) 0.2 M NaCl in 0.2 M HCl (ATP). Use of such a solvent system permits achieving a minimal intercontamination of the fractions and individual nucleotides are extracted with relatively small volumes. For regeneration of the resin the column is washed with M HCl, which carries along extraction of some quantity of nonidentified nucleotides (Fig. 2).

5-6 ml fractions were collected with an automatic collector.

The concentration of nucleotides in the fractions was determined by the optical density at 260 mμ, measured on the SF-4 spectrophotometer, using a 10 mm layer of solution. The optical density of rich nucleotide fractions was determined after dilution of the solutions 50 and 100 times and calculated by multiplying the magnitude of the optical density by the magnitude of dilution. The molecular extinction coefficient of adenosine at the given wavelength is 14,200 [8].

Results of chromatographic separation of a commerical preparation of ATP are given in the table (preparation date – March 1954).

The test conditions were as follows: Tests 1 and 2: ion exchange resin AV-16, 250 mesh. Cl^-, column 3 × 0.9 cm (internal diameter), flow rate 0.2 ml/min per cm^2. ATP – 20 mg. Test 3: ion exchange resin EDE-10-P, 250 mesh, Cl^-, column 3× 1.1 cm (internal diameter), flow rate 0.14 ml/min per cm^2, ATP – 40 mg.

As can be seen from the table, the variation in results of repeated determinations of the content of each nucleotide in a given ATP preparation did not usually exceed ± 10%.

From our data the ion exchange resins EDE-10-P and AV-16 are of equal value in nucleotide separation. EDE-10, AN-2F, AN-9 require a somewhat higher concentration of HCl and NaCl in the eluting solutions for separation, but they are also quite suitable for nucleotide separation. The sample of resin N-0 which we had available was indicated as being unsuitable for separation of adenosine phosphates.

Attempts to separate adenosine phosphate on various resin columns (AV-16, AN-2F, AN-9, EDE-10-P) in the formate form were unsuccessful. We were not successful in quantitative separation of the nucleotides into fractions by any of the solvent systems studied.

Preparative Separation of Adenosine 5'-Phosphate Esters

Commercial preparations of free ATP were used for the studies in most cases.

In treating adenosine phosphates with barium salts their conversion to the free acid was brought about by solution of a water suspension of the barium salt with a suspension of the KU-2 exchange resin (250 mesh) in the hydrogen form until the disappearance of the white precipitate, after which the resin was removed by centrifugation or filtration and washed with water.

The optimum load for a preparative column of the dimensions above-indicated was 0.5-1.0 g nucleotide mixture.

Results of Chromatography on Ion Exchange Resin of a Commercial Preparation of Free Adenosinetriphosphate (for test conditions see text)

Test no.	Content in %			
	AMP	ADP	ATP	nonidentified nucleotide
1	15.4	22.1	59.7	2.8
2	13.4	21.4	60.0	5.2
3	19.8	22.2	53.7	4.3

The adsorption of nucleotides was conducted as described above for the analytical separation.

Elution of the nucleotides from the column was carried out with solvents at a higher HCl concentration (due to the increase in NaCl concentration), and they were changed depending upon determination of the optical density of the fractions being removed. The volume of each fraction was ordinarily about 20-22 ml.

The following system of solvents was used: 1) 0.14 M NaCl in 0.01 M HCl (AMP); 2) 0.5 M NaCl in 0.01 M HCl (ADP); 3) 1.0 M NaCl in 0.01 M HCl (ATP).

In all cases, both the analytical and preparative separations, the total quantity of nucleotides eluted from a column amounted to 90-95% of those adsorbed on the resin.

Preparative Separation of Inosine 5'-Phosphate Esters

The materials investigated were solutions of inosine phosphates, prepared by diamination of adenosine phosphate by a slight modification of the Kleinzeller [5] method.

1 g of a commerical preparation of free ATP (or that obtained from the barium salt) was dissolved in 30 ml water and neutralized with 2 M NaOH. 4.52 g $CH_3COONa \cdot 3H_2O$ and 10 ml glacial acetic acid were added (pH of the mixture 4; glass electrode!). There is then added 20 ml of a 60% aqueous solution $NaNO_2$ and the mixture is allowed to stand 5-6 hrs at room temperature. After neutralization with 2 M NaOH (to pH 6-6.5) 12 ml 3 M $BaCl_2$ and 2 volumes of absolute alcohol are added. The solution is refrigerated overnight. It is then centrifuged 5 min at 2000 rpm, the precipitate washed 3 times with 50% ethanol, suspended in 50 ml water and 3 M HCl added to a final concentration of 0.1 M. After centrifugation the precipitate is washed with 0.1 M HCl, to the combined centrifugates is added 1 g urea and they are allowed to stand 2 hrs at room temperature. The solution is then made up to 200 ml and neutralized with 3 N ammonia until the appearance of a white turbidity. For removal of Ba ions the solution is shaken with a water suspension of the cation exchange resin KU-2 (250 mesh) in hydrogen form until the disappearance of turbidity.

The adsorption was carried out in the same manner as described above for separation of adenosine 5'-phosphate esters.

Elution of the nucleotides was carried out by use of the following solvents: 1) 0.14 M NaCl in 0.01 M HCl (IMP); 2) 0.5 M NaCl in 0.01 M HCl (IDP); 3) 1.5 M NaCl in 0.01 M HCl (ITP) (Fig. 3).

Separation of the nucleotides with a minimum carryover of impurities into the fractions with a low concentration of hydrochloric acid in the eluting solutions is achieved with this solvent system. However, as in the case of the preparative separation of adenosine nucleotides, fairly large volumes of solvents are necessary for elution of the individual nucleotides. The molecular extinction coefficient of inosine at 260 mμ was taken as 7400 [8].

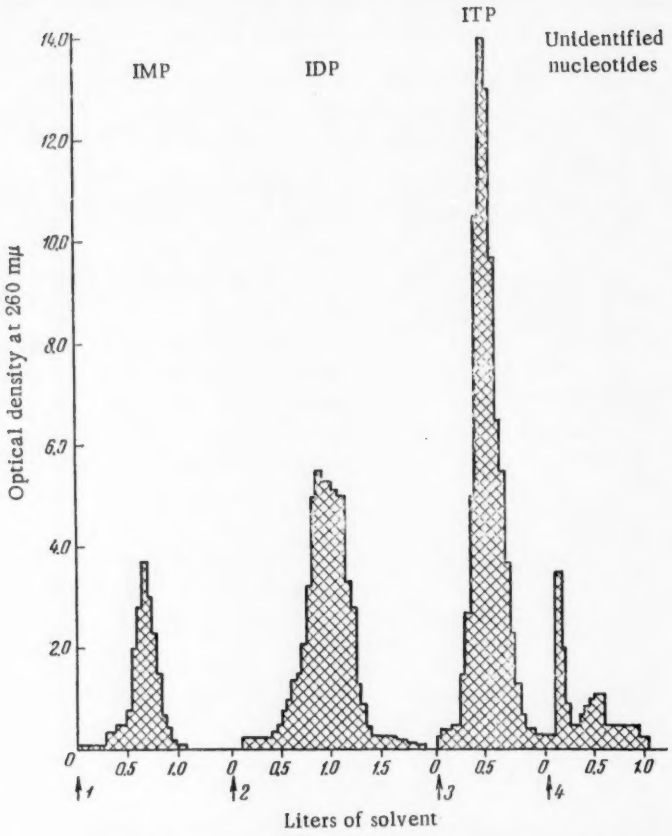

Fig. 3. Separation of preparative quantities of inosine 5'-phosphate esters: resin EDE-10-P, 250 mesh, 8.5 × 2.2 cm (internal diameter), Cl^-, flow rate 0.5 ml/min per cm^2, material investigated (deamination product of commercial preparation of ATP) – 480 mg inosine phosphate. 1) Beginning of elution of 0.14 M NaCl in 0.01 M HCl; 2) beginning of elution with 0.5 M NaCl in 0.01 M HCl; 3) beginning of elution with 1.5 M NaCl in 0.01 M HCl; 4) beginning of elution with M HCl. The total yield of eluted nucleotides – 100%.

Separation of Adenosine and Inosine Nucleotides from Dilute Fractions

Separation of adenosine and inosine nucleotides from the corresponding dilute emerging fractions was conducted by adsorption on activated carbon, from which the nucleotides were eluted by ammoniacal alcohol.

The brand of carbon ST (100-250 mesh), used in sugar production, was boiled 3 times with 3 M HCl for 1 hr with subsequent washing with several volumes of boiling water; it was then dried, again boiled with water, washed free of traces of acid and held at 150° for 48 hrs [9]. Immediately prior to the tests the carbon activated in this manner was again baked for 2 hrs.

To a dilute nucleotide solution 10 mg of carbon was added per mg nucleotide. The carefully mixed solution was allowed to stand 15-20 min at room temperature, after which it was filtered on a Buchner funnel. From the optical density of the filtrate the nonadsorbed nucleotide content was calculated and carbon was added to the filtrate at the rate of 20 mg per mg remaining nucleotides. Filtration was conducted through the same filter as used for the first filtration.

After washing of the carbon deposit with water to remove the main mass of Cl^- ions, the nucleotides were eluted in ammoniacal alcohol solution (ethanol – concentrated ammonia (sp. g. 0.88) – water = 25 : 1 : 24) [10],

in a volume equal to that of the volume of the deposit. After a sixfold extraction of the carbon precipitate in centrifuge tubes, extracts, always containing a thin carbon suspension, were filtered through layers of paper pulp or powdered cellulose [11] 2-2.5 cm deep. The completely transparent filtrates were concentrated under vacuum at 23-25° to $^1/_{10}$ - $^1/_{12}$ the original volume, and to the nucleotides precipitated in the form of barium salts was added 2 volumes of ethyl ether and 2 M barium acetate to complete precipitation. The precipitate was separated by centrifugation at 2000 rpm in 5 min, washed with alcohol and vacuum-dried over calcium chloride.

The total yield of separated adenosine and inosine phosphates relative to those obtained as a result of elution of pure nucleotides on the column was 50-60%, the main loss occurring as a result of incomplete elution of the nucleotides from the carbon.

Verification of Homogeneity of the Preparations Obtained

Verification of the homogeneity of the preparations obtained was conducted (after conversion to the free acid) by paper partition chromatography [12].

Sheets of "slow absorbing" chromatographic paper No. 4 measuring 19 × 30 cm produced by the Volodarsky Leningrad factory were immersed, according to the method suggested by Venkstern [15] for 15-20 min in 0.5% disodium ethylenediaminetetraacetate (Trilon B, Versene) at pH 8.5 at the rate of 0.5 liter solution per 10 sheets of paper (with twofold use of the solution). The paper was then washed with 2.5-3.1 freshly distilled water and dried at room temperature.

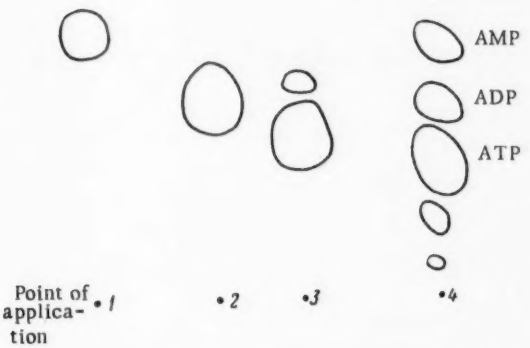

Fig. 4. Scheme of paper chromatography of adenosine 5'-phosphate esters: for conditions see text. 1) AMP (purified); 2) ADP (purified); 3) ATP (purified; 4) ATP (commercial).

The nucleotides were applied to the paper in a volume not exceeding 10-15 μ liters; their total quantity per sample was 200-300 μ g. Separation by descending partition chromatography by the solvents (n-propanol: concentrated ammonia (sp. g. 0.88): 0.002 M Trilon B = 60:30:10) [12] was conducted for 96 hrs. The paper was dried (after the first 48 hrs and after completion of chromatography) at room temperature. The position of the spots was determined using a quartz-mercury lamp, with a Wood filter (chemiscope) and a fluorescent shade.

As can be seen from Fig. 4, the purified preparation of nucleotides was almost entirely free of traces of other nucleotides. A small admixture of ADP in the purified ATP preparation in all likelihood forms during dephosphorylation of ATP in the course of treatment.

The results of our investigations of purified nucleotide preparations by paper electrophoresis were in accordance with these results [13].

The yield of individual nucleotides (calculated as free acid) in relation to the nucleotides contained in the fractions taken for their separation was as follows: AMP – 50%, ADP – 58%, ATP – 60%, IMP – 55.5%, IDP – 55%, ITP – 53.5%.

DISCUSSION OF RESULTS

As indicated by the data, both the analytical and preparative separation of adenosine and inosine phosphates can be carried out on the domestic anion exchange resins EDE-10-P and AV-16 in the chloride form. In contrast to the columns of Dowex-1 [1] and Dowex-2 [3, 4] solvents with high Cl^- concentration must be used for elution of nucleotides from the columns of EDE-10-P and AV-16. This attests to the more stable binding of the nucleotide anions by the domestic resins. Taking this into account, in the preparative tests the ionic strength of the eluting solutions was increased by increasing the sodium chloride concentration and not hydrochloric acid, so as to avoid hydrolysis of the labile phosphate esters.

Firmer binding of the inosine phosphate by the resin by comparison with adenosine phosphate was found in studies with Dowex-1 [14] and Dowex-2 [4]. This was also observed in our work, but it was less marked. In the tests of Deutsch and Nilsson [4] ITP was eluted from the Dowex-2 column with M HCl. In our tests with the EDE-10-P column an 0.01 M HCl in 1.5 M NaCl was used, in which the possibility of ITP hydrolysis was much less than in M HCl. In this regard EDE-10-P is a more convenient anion exchange resin than Dowex-2.

Upon elution from the columns there are obtained relatively large volumes of dilute nucleotide solutions. By adsorption on activated carbon with subsequent elution by an ammoniacal alcohol solution and concentration under vacuum, there are obtained concentrated nucleotides in small volume, after which the separation of the barium or calcium salts or the free nucleotides is not a difficult matter. According to the data in the literature [10] the loss of nucleotides upon elution from carbon reaches 20% of the adsorbed substance. In our tests this loss amounted to 30-35%. We could not find any explanation for this loss in the literature available to us; there are data only as to the relationship of the losses to the carbon variety.

Failure in the attempts to obtain a separation of adenosine phosphates on domestic anion exchange resins in the formate form indicates the need for further work in perfecting domestic anion exchange resins, in particular in obtaining an anion exchange material of the copolymer type with determination of the number of cross linkages.

SUMMARY

The technique of separation of adenosine and inosine phosphates by chromatography on Soviet anion exchange resins (EDE-10-P and AV-16) in the chloride form is described. For the elution of the nucleotides from the columns of these anion exchangers the use of relatively large concentrations of Cl^- (as compared with Dowex-1 and Dowex-2) is necessary. A description of the preparation of chromatographically pure samples of adenylic, adenosine diphosphoric and adenosine triphosphoric acids (and of the respective inosine derivatives) from the commercial ATP is presented.

We are very grateful to Professor A. E. Braunshtein, Member of the Academy of Medical Sciences USSR, for his constant attention and guidance in this work.

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A COMPARATIVE STUDY OF THE ELECTROPHORETIC AND IMMUNOLOGICAL PROPERTIES OF TISSUE AND SERUM PROTEINS

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It was shown in our previous communications [1, 2], dealing with the electrophoretic properties of rat and dog liver proteins, that the mobilities of a number of the protein fractions, as demonstrated with either the classical Tiselius method or by paper electrophoresis, coincided almost completely with the mobilities of some serum proteins. Of great interest also was the observation that the electrophoretic mobilities of some liver proteins corresponded to the mobilities of some serum proteins from embryonic, but not adult, blood [3].

In this connection there arose the problem of the similarity of some liver and serum proteins possessing the same mobilities, as well as other comparable properties. It was of interest to establish first whether there existed any immunological similarity between these proteins, since it has already been shown in this laboratory that adult and embryonic serum proteins exhibiting similar electrophoretic mobilities differed sharply in their immunological properties [4].

Very few references exist in the literature on the subject of comparative immunological properties of serum and tissue proteins [5, 6].

The most interesting results were obtained by Gitlin et al. [5], who showed that a certain number of proteins, immunologically identical with those from serum, could always be found among the liver and other tissue proteins. These data were obtained in the histological examination of tissues treated with fluorescent serum protein antibodies. However, the studies of Gitlin et al. provided no indication of the quantities of the various proteins present, nor did they solve the problem of their origin.

With these problems in mind, the present work was undertaken with the aim of a parallel study of the electrophoretic and immunological properties of some proteins from liver, various tissues, and from serum.

EXPERIMENTAL AND METHODS

The Preparation of Antigens for Animal Immunization

Serum from normal adult white rats was subjected to electrophoresis in starch blocks, with either veronal-medinal buffer (pH 8.6) or veronal-hydrochloric acid buffer (pH 9.2), for 30 hrs with a potential gradient 8 v/cm and current 15 ma. A small amount of Evans blue (T-1824), which stains albumins selectively, was added to the protein solution in order to enable us to observe the progress of the serum albumins. At the end of the electrophoresis, the starch block was sectioned across into strips 1 cm wide and the separate strips placed into test tubes and eluted with the same buffer which was used in the electrophoretic separation. The protein content of each tube was determined according to Lowry [7] and the total protein distribution in the starch block was then plotted. Using the plot, the samples representing the various fractions were combined. The purity of each fraction thus prepared was checked by paper electrophoresis.

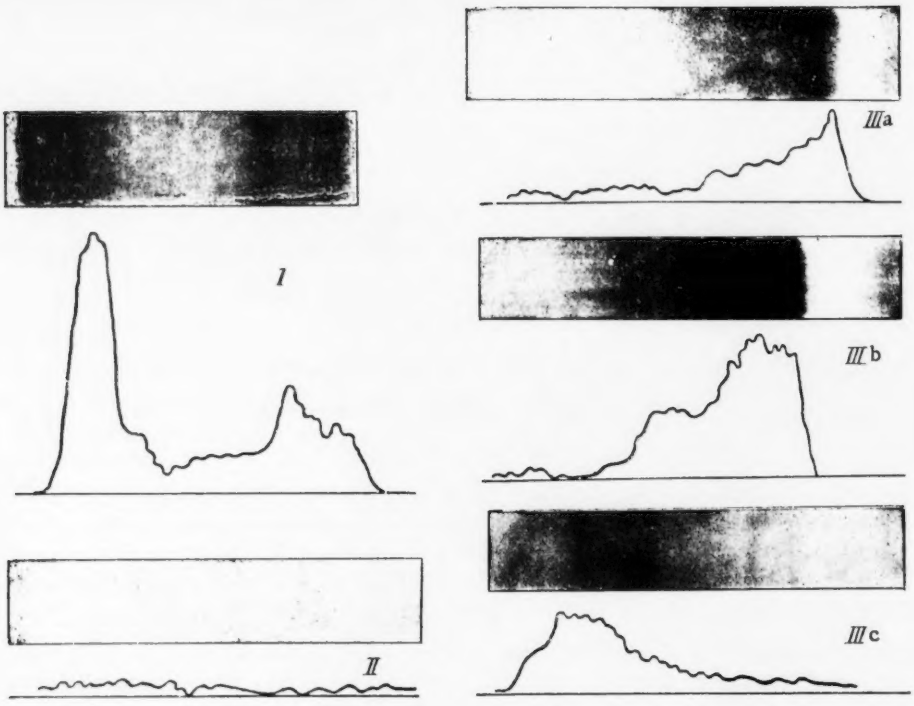

Fig. 1. Check on the purity of antisera by the electrophoretic precipitation method: I) rat blood serum electrophoregram; II) rat blood serum electrophoregram after elution in the chromatographic chamber; III) precipitates remaining after elution of the electrophoregram in the chromatographic chamber; a) in the region of γ-globulins; b) in the region of β- and γ-globulins; c) in the region of albumins.

Immunization of Animals

Electrophoretically pure proteins only were used for immunization. One of the isolated protein fractions was introduced over a period of 3 weeks, every second day, to adult rabbits weighing 2-3 kg. The doses of antigen administered were 1.0, 1.5 and 3.0 mg in the first, second and third week, respectively. Blood was drawn from the ear vein 7 days after the last immunization and its antibody content was determined by a modification of the immunochemical method developed in this laboratory [8]. The specificity of the antisera was checked using the ring precipitation and electrophoretic precipitation reactions [9]. Specific immune sera, containing antibodies against γ-globulin and serum albumin, were thus successfully prepared. In the case of β- and α-globulin antibodies it was found that the serum from rabbits which were immunized with the electrophoretic β- and α-globulin fractions contained, apart from the corresponding antibodies, also antibodies active against other protein fractions from serum. It may be seen from Fig. 1 that the antisera for albumin (IIIc) and γ-globulin (IIIa) reacted only with one corresponding serum fraction, while the immune serum obtained from the immunization with β-globulin reacted with the corresponding β- as well as with the γ-globulins (IIIb). These sera were therefore further purified by precipitation of the contaminating antibodies with the corresponding antigens. This was successful in the case of the β-globulin antiserum; however, even the additional purification step failed to produce an immune serum, selectively specific for α-globulins.

The antibody content in each antiserum was determined and a calibration curve was constructed for each preparation, where the size of the precipitate was expressed in terms of the amount of antigen added.

Preparation of Soluble Liver Protein Extracts

The rats were killed by decapitation, the peritoneal cavity opened and the livers were perfused with physiological saline solution through the portal vein. The livers were then washed free from blood, homogenized with an equal volume of physiological saline solution in a glass homogenizer, and the homogenate was frozen by triturating in a mortar with liquid air and left at −1° for 24 hrs. After thawing the homogenates were centrifuged, treated with ether and centrifuged again. The resulting extract of the soluble proteins was slightly opalescent, golden in color and contained about 4% protein. A portion of this extract was used for paper electrophoresis, which was carried out in borate buffer pH 8.68, in 20-hr runs with a voltage of 210 v and current 4.5 ma. After the run the papers were dried, dyed with the acid blue-black dye and passed through the densitometer [10]. From the curve obtained the percent composition of the protein fractions in the liver was calculated.

Immunochemical Determination of the Liver Proteins Identical with Serum Proteins

In order to detect in the liver extract proteins identical with those of serum, as well as to determine the amounts present, aliquots of the undilute extract, and of extract diluted 2 and 4 times with physiological saline solution, were mixed with equal volumes of the corresponding antisera and the size of the precipitate obtained was estimated. The determinations were carried out according to Peters and Anfinsen [8], with the difference that the protein in the precipitate was determined according to Lowry.

The quantity of proteins identical with the serum proteins was calculated by means of the calibration curve described above.

RESULTS AND DISCUSSION

The data obtained on the content of proteins, identical with serum proteins, in the various organs of the rat are shown in the table. Presented in Fig. 2 is the correlation of the various protein fractions from liver and serum, obtained by paper chromatography.

The Content of Proteins, Immunologically Identical with Serum Proteins, in Various Tissues

Electrophoretic fraction of the soluble tissue proteins	Serum protein fraction of corresponding electrophoretic mobility	Liver			Kidneys			Brain		
		individual fraction protein in mg per 100 mg of total protein	protein identical with serum protein		individual fraction protein in mg per 100 mg of total protein	protein identical with serum protein		individual fraction protein in mg per 100 mg of total protein	protein identical with serum protein	
			in mg/100 mg of total protein	in % of fraction protein		in mg/100 mg of total protein	in % of fraction protein		in mg/100 mg of total protein	in % of fraction protein
Immobile fraction	—	9.7	—	—	7.4	—	—	5.0	—	—
Fraction I	γ-globulin	25.0	1.43	5.7	37.0	1.92	5.2	33.0	1.9	5.8
" II	β-globulin	41.0	0.95	2.3	27.2	2.09	7.7	23.0	0.9	3.9
" III	α-globulin	18.5	—	—	22.4	—	—	31.0	—	—
" IV	Albumin	5.8	5.44	93.8	7.0	6.6	94.5	8.0	2.9	36.0

It is seen from the table that almost the entire liver fraction, corresponding in its mobility to the serum albumin, is immunologically identical with this protein. The content of the protein identical with serum albumin varied in individual preparations between 88 and 100%, the mean value being 93.8%. It is impossible to ascertain at present whether the small remaining fraction of the protein is immunologically distinct from serum albumin, or whether the discrepancy was due to experimental error.

In the liver protein fractions, corresponding in their electrophoretic mobilities to the serum β- and γ-globulins, the amount of protein, immunologically identical with the corresponding serum proteins, did not exceed 2.5% in the case of the β-globulins, and 6% in the case of the γ-globulins. The same held for the

kidneys. In this organ the amount of protein immunologically identical with the serum albumin fraction was equal to the total amount of albumin detected electrophoretically. The amount of protein immunologically identical with serum γ-globulin was approximately the same as in liver, while the content of protein identical to serum β-globulin was slightly higher.

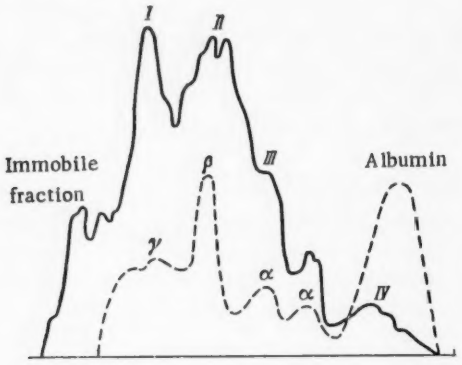

Fig. 2. Comparison of plots obtained from the electrophoretic separation of soluble liver proteins and of rat serum proteins. ——) Plot representing the separation of the soluble liver proteins; ----) plot representing the separation of serum proteins.

An entirely different picture presented itself in the case of brain albumins. The amount of cerebral protein, immunologically identical with the serum albumin was only 36% of the total albumin detected electrophoretically in the hemispheres. The quantities of proteins immunologically identical with the serum β- and γ-globulins were approximately the same as in the liver.

As a result of the present investigation into the presence, in various organs, of proteins immunologically identical with those of serum, it was found that their content in the various organs varied within wide limits. In view of the fact that the liver is, apparently, the only organ where serum albumin is synthesized [11], it may be confidently assumed that the albumin found in the present work in the liver, and identical immunologically with serum albumin, actually was serum albumin synthesized in the liver prior to its passage into the blood stream, its immunological and electrophoretic properties unaltered.

The results obtained with kidney extracts are of great interest. There are no indications at present about any synthesis of serum albumin that may take place in the kidney; however, almost the entire fraction of the kidney proteins, which corresponded in its electrophoretic mobility to serum albumin, was also immunologically undistinguishable from the latter. This forces the conclusion that this fraction arose due to the penetration of unaltered serum albumin from the blood into the kidneys.

The fraction of brain protein electrophoretically similar to the serum albumins may be subdivided,on the strength of the results obtained, into two sub-fractions. The first of those, and quantitatively the smaller, was identical immunologically with the serum albumin fraction and its presence may also be explained by the penetration of unaltered serum albumin into the brain tissue; the second fraction was found to be immunologically distinct from the albumins and is, possibly, synthesized in the brain tissue itself. Further investigations are necessary in order to resolve the problem of the origin of these two fractions.

As shown above, only a small amount of the liver protein was found to be immunologically identical with the serum β-globulins. The fraction II of the tissue proteins also consists of a number of sub-fractions. Some of those differ slightly in their mobility from the serum β-globulins. However, the largest of these sub-fractions, comprising 50% of the total fraction, corresponds well in its mobility with the serum β-globulin fraction. Nevertheless, when tested immunologically, only 5% of this large sub-fraction was found to be identical with serum β-globulin.

The presence in the liver of proteins, immunologically identical with the serum β-globulins, could be due to both β-globulin synthesis in the liver and to the penetration of the protein from the blood stream into the tissue. In the kidney and brain, where the probability of serum β-globulin synthesis is probably excluded, the presence of proteins immunologically identical with those of serum is, apparently, due to penetration from serum alone.

The relative content of proteins immunologically identical with those of serum was also found to be low in all the tissues investigated.

Specially conducted experiment in this laboratory, dealing with the incorporation of labeled amino acids into specific and nonspecific serum γ-globulins in hepatectomized animals, showed that hepatectomy did not affect labeled amino acid incorporation into the nonspecific serum γ-globulins, or into the specific antibodies.

This supports the view that the liver is of little importance in the synthesis of the nonspecific serum globulins, and indicates the small role this organ plays in the process of antibody production. The presence in the liver of proteins, electrophoretically and immunologically similar to serum γ-globulins is, apparently, due to the penetration of these proteins from the blood. Those liver proteins which are electrophoretically but not immunologically similar to the serum γ-globulins are most probably distinct liver proteins of various physiological functions, and their relationship with immunity bears further investigation.

The demonstration of the presence in various organs of protein fractions, immunologically and electrophoretically identical with serum proteins, irrespective of whether these proteins are actually synthesized in the given organ, confirm the belief about the existence of a dynamic equilibrium between the soluble protein of the various organs and those of serum. It may be expected that a disturbance of this equilibrium, in the case of damage to liver, kidney or brain, will be followed by considerable changes in the distribution in the tissues of proteins identical with those of serum.

The study of this problem is of great interest in the elucidation of the origin of changes in the composition of serum proteins in various states of the body, and investigations in this direction are presently being conducted in this laboratory.

SUMMARY

It was shown, using quantitative immunological methods, that almost the entire (93-100%)protein fraction of the rat liver and kidney, which showed electrophoretic mobilities similar to those of serum albumin, was also immunologically identical with the latter.

Only one-third of the similar proteins in brain was found to be immunologically identical with serum albumin.

Unlike the albumin fraction, only a small portion (3-7%) of the liver, kidney and brain protein, electrophoretically similar to the β- and γ-globulins of serum, was found to be identical with them immunologically.

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THE EFFECT OF X-RAYS ON THE ENZYMATIC SYSTEM OF COENZYME A OF THE PIGEON LIVER

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Considerable progress has been observed in the recent years in the fields of biochemical research connected with the study of coenzyme A function.

New facets of metabolism where this coenzyme participates are continuously being discovered. Its role has now been demonstrated in oxidative substrate catabolism, in the generation of energy necessary for cell function and in the synthesis of a variety of cellular components, some of them of high complexity. Although the function of coenzyme A is far from being completely outlined, there is sufficient evidence to consider this compound as one of the central factors in metabolism, since it participates in the most fundamental reactions and processes underlying life itself.

If the importance of coenzyme A (CoA) has not yet been completely elucidated in the normal cell, the understanding of the processes in which it participates in pathological states is even less complete. Yet it seems clear that interference with such important a function in the cellular metabolism, as that of CoA and related systems, may determine the origin, progress and outcome of many diseases.

Of great interest in this connection is severe radiation syndrome. All the known effects of this severe and complex pathological condition point to a serious disturbance of both assimilatory and dissimilatory phases of metabolism. The radiation-induced disturbances include both the energetic and plastic metabolism, and appear to be due not only to the change in some reactions and processes, but to the interference with their coordination.

Such total effect of radiation on the living cell prompted a search for an effect upon some factors which would possess an integrating function in metabolism. One such factor is CoA, which integrates a number of facets of cellular activity.

The present communication deals with an investigation into the effect of total body x-irradiation on the activity of CoA and its enzymatic system in the pigeon liver.

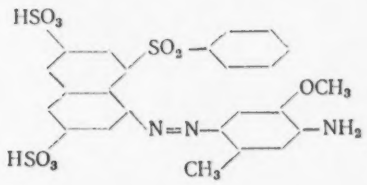
EXPERIMENTAL

The investigation comprised a study of the acetylating function of the CoA enzymatic system, as well as of the changes in coenzyme concentration in the pigeon liver during severe radiation syndrome caused by the action of x-rays.

The birds were irradiated with a single dose of x-rays, 2 to 3 thousand r. Symptoms of severe radiation sickness appeared 5-7 days after irradiation and the pigeons died on the 8th-9th day. All investigations were carried out in the period between the 5th and 7th day. At that stage the irradiated pigeons showed a decreased leucocyte count in the peripheral blood, the value falling from approximately 25,000 per μ liters to 2,000-9,000, disturbances of the alimentary tract, loss of appetite and loss of weight. The leucocyte count and the weight of the animals was checked before irradiation and directly prior to experiment.

Acetylating activity was measured both in liver homogenates [1] and in autolyzed extracts of acetone powders, prepared from liver according to Kaplan and Lipmann [2]. The acceptors of activated acetate, employed

in the work, were: sulfanilamide (SAM), p-aminobenzoic acid (PABA) (the method of Bratton and Marshall [3] as modified by Sytinskaia [4]), p-aminoazobenzene [5], 4-amino-1,1'-azobenzene-4'-sulfonate sodium salt (PAABS) and, for the first time employed in this work, a dye obtained by coupling cresidine with acyl-H-acid (K)*:



The last two compounds are particularly convenient in that they lose their color on acetylation (the former is amber, the latter crimson) and their concentration may be determined photometrically in the trichloroacetic acid filtrate without further treatment.

TABLE 1

Sulfanilamide Acetylation in Liver Homogenates from Normal and Irradiated Pigeons

Starting amounts SAM – 1000 μg. Incubation 20 min, temperature 37°. All values calculated per g of fresh liver.

Control pigeons		Irradiated pigeons		
leucocyte count per μ liters blood	SAM acetylated in μg	leucocyte count per μ liter blood: before irradiation	leucocyte count per μ liter blood: at time of expt.	SAM acetylated in μg
	540			160
	390			145
	540			128
	490			90
	400			54
	440			0
	340			62
	340	25 040	6220	104
24 200	560	23 760	8400	71
25 120	420	26 100	9240	117
21 560	500	21 150	5980	160
25 000	500	23 600	9400	180
27 000	530	24 800	5340	116
25 600	420	21 200	5980	220
26 700	600	22 200	5220	160
24 520	180	26 800	2100	150
23 700	450	20 110	1800	107
23 300	620	22 760	2740	90
24 030	460	22 500	2480	107
24 480	530	20 900	3280	117
21 400	510	25 600	2940	107
		24 880	14 220	180
Mean	465±16			119±8.5

For the preparation of homogenates the pigeons were bled by cardiac puncture (12-16 ml of blood was collected) and decapitated, the liver was then rapidly excised, cooled and homogenized in the cold with 1.5 volumes of 0.04 M K-phosphate buffer pH 7.6 (with the addition of KCl and $MgCl_2$ [1]).

* The authors wish to thank M. S. Dinaburg for assistance in the preparation of this compound.

TABLE 2

Acetylation of p-Aminoazobenzenesulfonate and Dye K by Liver Homogenates of Normal and Irradiated Pigeons

Acetylation in μg/g of tissue (20 min)	
Control pigeons	Irradiated pigeons
p-Aminoazobenzenesulfonate	
600	0
960	94
870	95
640	0
460	185
470	210
Dye K	
150	70
151	71

TABLE 3

The Effect of Excess Coenzyme A on Sulfanilamide Acetylation by Liver Homogenates of Normal and Irradiated Pigeons
All values as μg acetylated SAM per g tissue in 20 min.

Control pigeons		Irradiated pigeons	
homogenate alone	homogenate + CoA	homogenate alone	homogenate + CoA
450	450	216	390
630	650	180	532
460	490	160	260
520	560	150	270
510	570	109	230
—	←	90	135

To each ml of homogenate was then added 0.1 ml Na acetate (0.025 M), 0.1-0.2 ml of acceptor solution and the mixtures were incubated in Warburg vessels for 20 min at 37°, after which they were treated with trichloroacetic acid and the decrease in free acceptor was measured in the supernatant after centrifugation.

The results of a number of experiments, in which SAM was used as the acceptor, are shown in Table 1.

It will be seen from Table 1 that SAM acetylation by liver homogenates was considerably inhibited in the irradiated animals compared with normal controls. In the former case, on the basis of 1 g fresh tissue, 119 ± 8.5 μg SAM was acetylated, while in the second case the value was 465 ± 16.

Identical results were obtained with the other acceptors.

The results of a number of experiments, using PAABS and the dye K as acceptors, are shown in Table 2.

There was also quite pronounced inhibition of acetylating activity in liver homogenates from irradiated pigeons when it was assayed using PABA and PAAB as acceptors.

The good agreement of experimental results in the case of the different acetyl group acceptors indicated that irradiation damage had been sustained by the acetylating system of CoA under the conditions prevailing in the intact animal. However, since this system represents a complex, comprising a number of enzymes and coenzymes, experiments with the whole animal gave only an overall result, without the indication as to which particular link may be the most labile.

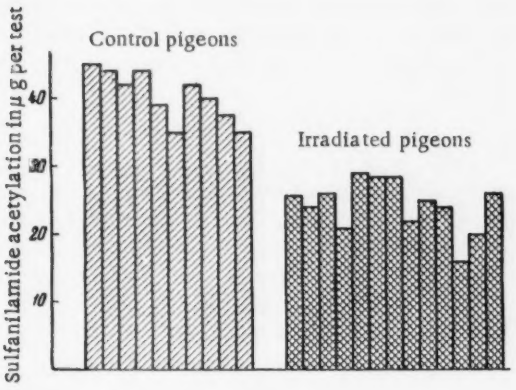
In an attempt to localize the effect of radiation, further experiments were carried out, in which excess of pure CoA (prepared in this laboratory) was added to the liver homogenates and the maximal acetylation rates were measured.

The addition of excess CoA to normal liver homogenates (cf. Table 3) was practically without effect on the acetylation rates observed, while in the enzymatic system of the liver homogenate from irradiated animals this treatment brought about an increase in acetylation rates without, however, raising the corresponding values to normal levels.

Although the results obtained (cf. Table 3) indicated the deficiency of CoA in the livers of irradiated pigeons, they also pointed to the loss in activity of the protein component of the enzymatic acetylating system, since the inhibition could not be completely reversed by excess CoA. A characteristic feature of this experimental series was the tendency of the reversing action of excess CoA to decrease with increasing severity of radiation sickness symptoms in the given animal and the concomitant decreased basal acetylating activity in the liver homogenates. This phenomenon gave cause to believe that, with increasing severity of radiation syndrome, the main reason for loss of acetylating activity in the liver is not so much the loss of coenzyme A, as the damage to the protein catalyst.

Since the results quoted above provided evidence about the alteration of both the coenzyme and enzymatic components of the acetylating system in the livers of irradiated pigeons, it was decided to isolate and investigate each of these components independently.

The enzymatic component of the acetylating system was studied in autolyzed extracts of pigeon liver acetone powders, prepared according to Kaplan and Lipmann [2]. CoA is lost from these extracts as a result of autolysis and they are quite inactive without the addition of CoA. Upon the addition of CoA, the condition of the enzymatic component in the acetate transfer system may be determined from the maximal rates observed.



Maximal acetylation rates, in the presence of coenzyme A excess, in enzymatic systems prepared from livers of normal and irradiated pigeons. Enzymatic system employed: autolyzed extracts of pigeon liver acetone powders + ATP + sodium citrate + sodium acetate + sulfanilamide + cysteine + $NaHCO_3$ (cf. [2]) + 20 units of CoA. Total volume 1.03 ml; 37°, incubation 120 min.

The study of the protein component of the acetylating system showed that the activity of the enzyme from the livers of irradiated birds was well below that of normal controls. On no occasion was it possible to raise acetylation values in the system from irradiated birds to normal levels by the addition of excess CoA alone.

The results of such an experimental series are shown in the figure.

It should be particularly noticed that not in one single case did the maximal acetylation level observed with the enzyme from irradiated pigeon livers reach the lowest value observed in similar preparations from normal livers. The overall mean data from the series were as follows: control preparations acetylated 38 ± ± 2.9 μg SAM per test; the corresponding value in preparations from irradiated birds was 26 ± 0.9 μg.

These results, together with those from the experiments dealing with the addition of excess CoA to whole homogenates, allow the conclusion that total body irradiation brings about a considerable decrease in activity of the enzymes concerned with acetylation, the effect being dependent on the severity of the damage.

It is however, known that the process of acetylation is brought about through the action of more than one enzyme and that it consists of at least two reactions, each catalyzed by its specific enzyme (or enzymes). The first stage – acetate "activation" – consists of the inclusion of the acetyl radical into the molecule of coenzyme A and is characterized by raising the energetic potential of the acetylated CoA derivative to the level of high-energy compounds; the second stage of the acetylation process is the transfer of the "active" acetate onto the respective acceptor. In the presence of hydroxylamine, and under suitable conditions, this latter reaction may take place not through enzymatic catalysis, but in a direct chemical reaction. Acetylhydroxamic acid is formed. This fact was utilized in the present work to distinguish, under the conditions of irradiation, between the "acetate-activating" and the "transacetylase" enzymatic systems.

Comparisons were carried out of the rates of SAM acetylation and acetylhyroxamic acid formation in liver homogenates from normal and irradiated pigeons. It was found (cf. Table 4) that in liver homogenates of normal birds the amount of acetylated SAM produced was always practically equivalent to the amount of acetylhydroxamic acid formed: 2.6 ± 0.07 μmoles of SAM was acetylated for each 2.4 ± 0.04 μmoles of acetylhydroxamic acid formed in parallel experiments. However, in preparations from irradiated birds acetylhydroxamic acid formation was always higher than SAM acetylation: under identical conditions 0.74 ± 0.1 μmoles of acetylated SAM, and 2.04 ± 0.19 μmoles of acetylhydroxamic acid, was formed.

Since the entire acetylation process is made up of two steps, and since in irradiated birds the only affected reaction seems to be SAM acetylation, but not hydroxamic acid formation, it may be deduced that the reaction blocked in this case is not acetate activation, but the subsequent acetyl transfer from coenzyme A to the acceptor. This follows from the fact that, in the formation of acetylhydroxamic acid, the first step, identical with the first step in SAM acetylation, includes enzymatic acetate activation, while the second step, independent of any enzyme, is unaffected by radiation.

TABLE 4

Sulfanilamide Acetylation and Acetylhydroxamic Acid Formation in Liver Homogenates from Normal and Irradiated Pigeons

Control pigeons		Irradiated pigeons	
SAM acetylation	acetylhydroxamic acid formation	SAM acetylation	acetylhydroxamic acid formation
3.5	3.0	0.5	2.2
1.8	2.0	1.2	1.8
3.0	2.8	1.3	2.5
2.3	1.8	0.3	2.5
1.5	1.9	1.2	1.5
3.7	2.7	0.5	1.8
2.9	2.6	0.7	1.9
2.8	2.5	0.6	1.7
2.9	2.5	0.4	2.0
2.0	2.0	0.7	2.5
Mean			
2.6±0.07	2.4±0.04	0.74±0.1	2.04±0.19

Note: Acetylhydroxamic acid was estimated according to the procedure of Lipmann [6] as modified by Beinert [7].

Thus, the effect of irradiation on the enzymatic component of the acetylation system has been exactly localized. The most labile link in this system seems to be the enzyme transacetylase.

As has been mentioned above, the experiments with whole homogenates revealed a deficiency of CoA in the livers of irradiated pigeons. In this connection attempts were made to characterize quantitatively this effect.

Boiled liver extracts (1 g of tissue + 3 volumes of water) of normal and x-irradiated pigeons were prepared and their CoA content was determined by testing the ability of each extract to stimulate acetylation in dialyzed acetone powder extracts of pigeon livers. A unit of coenzyme was taken to be that amount which produced a stimulation equal to 50% of maximal acetylation rates obtained with excess CoA [2]. Normal pigeon livers contained amounts of coenzyme equal to 100 tolerance units per g, while the corresponding value in livers from irradiated birds was only 67 units. Under the conditions of the assay 50% of maximal acetylation rates were achieved by the addition of 0.04± 0.002 ml of boiled normal liver extract, or of 0.06 ± 0.005 ml of the boiled extract from irradiated birds.

DISCUSSION OF RESULTS

The effect of ionizing radiation on the acetylating activity of the CoA system has not been thoroughly studied up to date. Very few reports are available on the subject.

It was shown by Trufanov and Popova [8] that coenzyme A biosynthesis was strongly inhibited in the brain of guinea pigs after x-irradiation with 500 r. Different results were obtained by Dubois et al. [9]. According to the latter authors, irradiation of rats with doses 400-800 r, and even up to 1200 r, had no effect upon the acetylation of SAM given intraperitoneally. Similar results were obtained with guinea pigs. The authors concluded that coenzyme A did not suffer in irradiation. It has been mentioned in a recent report by Romantsev and Zhulanova [10] that, following irradiation of rats with x-rays in doses 650 r, acetylation processes remained unaffected throughout the period of radiation syndrome. The assay system in the latter work involved the estimation of free and acetylated SAM excreted in the urine. The problem of the effect of radiation on the coenzyme A system remained thus unsolved.

The experiments quoted in the present work, dealing with the acetylating activity of liver preparations from normal and irradiated pigeons, allow the conclusion that ionizing radiation has, indeed, a pronounced effect upon the system concerned with the transfer of acyl groups. The effect was found to involve both the enzymatic and coenzyme components of the catalytic acetylation system. The result of irradiation is not merely a fall in coenzyme A concentration in the liver, but also an inhibition of the activity of the enzymes participating in acetyl group transfer. It may be proposed that the effect of ionizing radiation on the coenzyme A system plays an important role in the development of radiation sickness.

SUMMARY

It was shown that, 6-7 days after irradiation of pigeons in doses 2-3 thousand r, the intensity of acetylation processes in liver homogenates fell from a mean normal value of 465 ± 16 μg sulfanilamide per g fresh tissue in 20 min to a corresponding value of 119 ± 8.5 μg. Inhibition of acetylation has also been demonstrated with other acetyl acceptors: p-aminobenzoic acid, p-aminoazobenzene, p-aminoazobenzenesulfonate and a dye obtained by coupling cresidine with acyl-H-acid.

The general effect of inhibition of the acetylation system is due to the decrease in coenzyme A concentration in the liver (from 100 units per g liver in normal birds to 67 units after irradiation) as well as to the decrease in activity of the protein component of the system. The degree of inhibition of the enzymatic component of the acetylating system has been correlated with the severity of the radiation syndrome.

The results obtained indicate that the labile link in the system, affected by the radiation, is the catalytic reaction of acetyl group transfer from coenzyme A to the acceptor, that is, the enzyme transacetylase.

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ISOLATION FROM MITOCHONDRIA OF AN ENZYME WHICH CATALYZES AMINO ACID SYNTHESIS FROM PYRUVIC ACID AND AMMONIA AND ITS PURIFICATION BY ELECTROPHORESIS ON STARCH

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It was shown in our previous report [1] that the enzyme system present in rat liver and responsible for catalysis of amino acid synthesis from pyruvic acid and ammonia is located in the mitochondria. It was established that the liver cell nuclei contain a factor which stimulates the amino acid synthesis in mitochondria and some conditions of this synthetic process were explained.

Further investigation of this little-known enzyme system was hampered by the fact that the separation of it from the structural cell elements and preparation of the enzyme solution have not been achieved. In view of this, an attempt was made to put the above enzyme system in solution using various methods for the disintegration of liver cell mitochondria. If this proved successful the preparation could be purified and its properties and structure thoroughly investigated.

Results of the corresponding experiments are reported in this article.

EXPERIMENTAL METHODS

Fractionation of rat liver tissue as well as the determination of the activity of the enzyme system which catalyzes the synthesis of amino acids from pyruvic acid and NH_3 were carried out according to methods described in our previous report [1]. The separated mitochondria were suspended in 0.85% KCl solution (the total volume of mitochondrial suspension obtained from 5 g of liver – 9-10 ml) and subjected to disintegration by the following means.

1. Mitochondrial suspension was homogenized with 5 volumes of water for 10 min.

2. Mitochondrial suspension was homogenized with cooling for 2 min using various volumes of butyl alcohol [2].

3. Mitochondrial suspension was alternately frozen and thawed in a mixture of ice and salt.

The fragments of mitochondria disintegrated by these methods were separated by centrifugation (12,000 rpm for 15 min) and the enzymatic activity of the precipitate as well as that of the supernatant was determined by the increase in amino acids upon incubation with pyruvic acid and ammonia (for test setup see [1]).

RESULTS

Table 1 shows data on the extent of amino acid synthesis in mitochondrial extracts and fragments obtained by various methods.

It can be seen from Table 1 that mitochondrial extracts were completely inactive when mitochondria were disintegrated by prolonged homogenation in water or in butyl alcohol. Activity was observed in those extracts

TABLE 1

Increase NH_2–N in Extracts and Precipitates of Mitochondria Disintegrated by Various Methods (increase NH_2–N in moles per mg N)

Preparation method	Intact mitochondria	Extract	Precipitate
Homogenation with 5 volumes of water for 10 min	—	0	0
	—	0	0
	—	0	0
Homogenation with butyl alcohol for 2 min	—	0	0
	—	0	0
	—	0	0
Twice freezing and thawing	1.4	1.8	0
	0.9	2.5	0
	3.0	2.6	0.93
	2.7	2.6	0
	2.3	2.2	0
	1.1	2.5	0
	—	2.0	0.64

TABLE 2

Increase NH_2–N in Extracts of Mitochondria Disintegrated by Freezing and Thawing in the Presence of Nuclei (increase NH_2–N in moles per mg N)

Extract	Extract + nuclei
1.8	—
2.5	13.6
2.6	—
2.6	2.0
2.2	9.4
2.5	9.7
2.0	4.7

which were obtained by centrifugation of thawed mitochondrial suspension after the mitochondria were disintegrated by alternate freezing and thawing carried out twice. The precipitates were mostly inactive and only in a small number of experiments demonstrated slight activity.

On the average, the extracts contained 0.75 mg N in 1 ml; i. e., 0.5% of protein. As can be seen from Table 1 the extract activity when expressed in terms of mg N was equal to and in some experiments twice as great as that of intact mitochondria. It seemed interesting to determine whether the liver cell nuclei would have the same stimulating effect on the enzyme system in solution as they showed on the enzyme system of intact mitochondria.

In order to do so a number of experiments were performed in which a suspension of liver cell nuclei in 0.85% KCl solution (the total volume of nuclei suspension obtained from 5 g of liver – 8 ml) was added to the extract of disintegrated mitochondria being incubated with pyruvic acid and ammonia.

It can be seen from Table 2 that the addition of nuclei caused a considerable increase in the intensity of amino acid synthesis by mitochondrial extracts. In some of the experiments the increase in amino acid content in the tests containing the suspension of nuclei was 4-5 times greater than that in tests without nuclei. It was, therefore, shown that the liver cell nuclei factor exerted a greater stimulating action on the enzyme system in solution than on the enzyme system in intact mitochondria. In the latter case the increase in amino acids on the average doubled upon the addition of nuclei [1].

It follows from the above facts that we demonstrated for the first time the possibility of amino acid synthesis in cell-free preparations obtained from rat livers. This made it possible to proceed with the purification and separation of active proteins using electrophoresis or salting-out methods for protein separation.

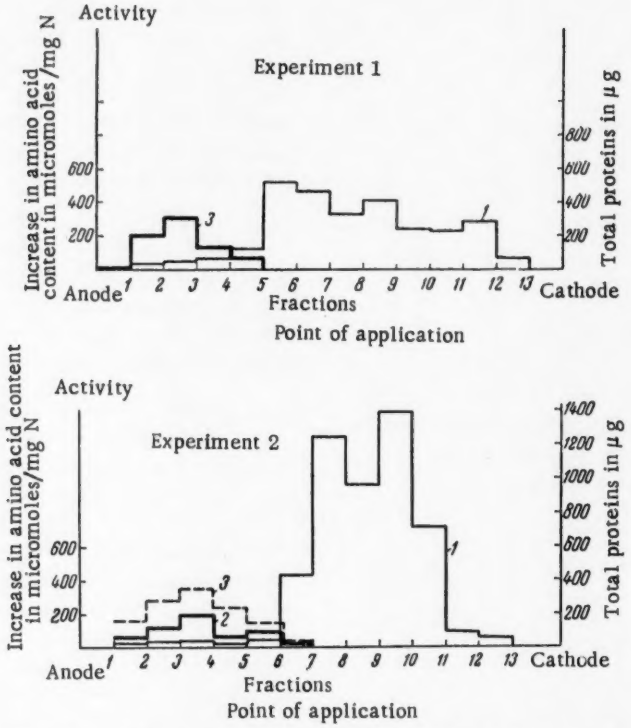
In this article results of the electrophoretic separation on starch of the extract proteins whichwere prepared from a suspension of mitochondria disintegrated by freezing are reported.

Electrophoresis was carried out in 0.05 M potassium phosphate buffer, pH 7.4. The time period of electrophoresis was 16-18 hrs at an approximate potential gradient of 6 v/cm and a current of 20 ma. In order to obtain an extract with a high enough protein content to be separated on starch, the mitochondria used in each experiment were prepared from livers of four rats and suspended, prior to their freezing, in a small volume of the same buffer solution in which the electrophoresis was carried out. The average protein concentration in the extracts prepared under these conditions was about 1%. The volume of the extract used in individual experiments was equal to 6-8 ml (i. e., 60 to 80 mg of protein was deposited on starch).

In some of the experiments the fractionation of liver tissues was carried out in sucrose solutions prepared in the same phosphate buffer in which the electrophoresis was carried out; in several of the experiments KCl was added to this buffer so that its concentration represented 0.85% (corresponding to the concentration of the sucrose solution which was usually used for the fractionation of the tissues). When the electrophoresis was finished, starch was cut transversely into 2-cm wide blocks. Each block was placed in a wide test tube and the proteins

extracted from starch with 6 ml of potassium phosphate buffer containing KCl. All of these operations were performed in the cold. Altogether 13 fractions were obtained, because the length of the starch column was 26 cm.

The extracts were filtered separately from each test tube, their protein content was determined according to the method of Lowry [3] and the activity of the enzyme by the increase in amino acid content upon incubation with pyruvic acid and NH_3. The increase in amino acid content was expressed in micromoles per 1 mg N (proteins present in small amounts in starch were thus taken into account).



Distribution of total proteins and of the enzyme which catalyzes the synthesis of amino acids from pyruvic acid and NH_3, upon electrophoresis on starch of extracts prepared from the liver cell mitochondria: Experiment 1) extracts do not contain KCl; Experiment 2) extracts contain KCl. 1) Total protein content; 2) activity expressed as the increase in amino acid content of the extracts; 3) the same as 2) but upon the addition to the extracts of the nuclei suspension.

The figure represents the data of the protein distribution according to fractions for two typical experiments with the extracts (Experiments 1 and 2) prepared by the first (without KCl) and second (with KCl) methods.

As can be noted from the curves the active protein is present in the rapidly moving protein fraction and has a definite peak, which in almost all of the experiments is located in a definite segment of the starch column usually corresponding to the fourth* consecutive fraction from the anode. At the same time an interesting phenomenon was observed; namely, the activity of this protein fraction from extracts prepared by the first method, i. e., without KCl, was exhibited only upon the addition to the incubated pyruvic acid of the nuclei suspension which contained the factor stimulating amino acid synthesis. When the suspension was not added the protein was inactive and no increase in amino acid content took place. At the same time the mitochondrial extracts prepared

* In Experiment 1 – the third, which is an exception.

by means of fractionating the liver tissues in the presence of KCl were active even in the absence of nuclei. Addition of nuclei in this case (see Experiment 2) enhanced the increase in amino acid content, i. e., stimulated their synthesis. The character of the distribution of proteins in extracts without and with KCl is dissimilar: in the first case, as it was to be expected, the separation was better and three separate peaks can be seen on the curves. Proteins in extracts containing KCl were poorly separated and only two peaks could be found on the curves. In both cases, however, the separation of the active protein from the concomitant ones took place to the same degree and its movement during electrophoresis was identical. The mode of action of potassium chloride upon the investigated enzyme system is not clear and is the subject of further investigation.

In several experiments rat serum was deposited on starch and subjected to electrophoresis under conditions strictly identical to those used for the separation of extract proteins. It appeared that the active protein which interested us represented a fraction which moved faster than serum albumin. When expressed in mg N, the increase in amino acid nitrogen in extract from starch block containing highest activity reached 300-360 micromoles in separate experiments. Since the activity of the initial liver homogenate used in our experiments represented about 1 micromole per mg N, consequently 300-350-fold purification of the enzyme was achieved on starch.

SUMMARY

It was demonstrated that synthesis of amino acids from pyruvic acid and ammonia can be accomplished in extracts prepared from disintegrated mitochondria of the rat liver cells subjected to alternate freezing and thawing.

Upon separation of extract proteins by means of electrophoresis on starch (phosphate buffer, pH 7.4), the active protein is contained in the rapidly moving fraction which has a higher mobility than the albumins of the blood serum. Electrophoresis resulted in a 300-350-fold purification of the active protein as compared with that of the original homogenate.

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AMINO ACID COMPOSITION AND TERMINAL AMINO ACIDS OF α-CRYSTALLIN

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Although the amino acid composition of protein preparations of crystallins was previously determined [1, 2], the reported data cannot be considered final because the purity of the investigated preparations was not checked. In 1954 the author was able to obtain from the lenses of cattle a homogeneous preparation of α-crystallin [3].

This investigation reports data about the amino acid composition and terminal amino acids of α-crystallin. The amino acid composition of this protein was determined by the Braunitzer modification [5] of the Levy method [4]. This method is based upon the chromatographic separation of dinitrophenyl derivatives (DNP-derivatives) of amino acids. A lyophilized preparation of α-crystallin prepared from lenses of cattle was used in this investigation. The purity of the preparation was checked by electrophoresis and by ultracentrifugation [6].

EXPERIMENTAL

Five mg of protein was hydrolyzed in a sealed ampule for 24 hrs using 0.5 ml of 6N HCl. The hydrolyzate was transferred to a wide test tube equipped with a ground glass stopper, and was made alkaline by the addition of 3 ml of 0.2N NaOH and 250 mg of dry sodium bicarbonate. Then 5 mg of dinitrofluorobenzene (DNFB) dissolved in 1 ml of ethanol was added to it. The test tube was tightly stoppered, placed in a constant temperature bath at 38° and kept for 2 hrs with constant shaking.

Extraction of DNP-amino acids. Prior to the extraction of the DNP-amino acids from the reaction mixture, the excess DNFB was removed by extracting with ether. Then the solution was acidified with 1 ml of 4N HCl and the DNP-amino acids extracted with several small portions of ether. With the exception of arginine, histidine and cysteine which remained in the aqueous fraction, the other DNP-amino acids were removed by ether extraction. Ether was removed by distillation and the dry residue dissolved in acetone in order to be applied to the chromatogram. In order to extract the DNP-amino acids which remained in the aqueous fraction, the latter was worked up with several portions of butanol. The alcohol was then removed by evaporation at 60-70° and the dry residue dissolved in acetone to be applied to the chromatograms.

Chromatography was carried out on Leningrad paper. The amount of hydrolyzate corresponding to 1 mg of protein was applied to the chromatogram. Each hydrolyzate was analyzed on two sheets. The first solvent used was butanol saturated with 0.1% ammonia solution (1 : 1), and the second solvent was 1.5 M phosphate buffer, pH 6. The DNP-amino acid spots were cut out of the chromatogram and eluted with 4 ml of 1% $NaHCO_3$ solution. Tests were kept for 15 min at 60° and then for 15 min at room temperature. Determinations of optical density of the solutions were carried out in a SF-4 spectrophotometer at 369 millimicrons (380 millimicrons for the determinations of DNP-proline solutions). Optical density of solutions containing original paper was also measured (control test) and its value was used as a correction for the colored tests. The optical density of the controls was: 0.018; 0.035; 0.025; 0.040.

DNP-amino acids of the aqueous fraction were determined on a one-dimensional chromatogram. Isoamyl alcohol saturated with a phthalate buffer, pH 6 was used as the solvent; the paper was treated with this solution prior to its use.

Tyrosine and tryptophan were not analyzed by means of the dinitrophenyl method but were estimated spectrophotometrically in unhydrolyzed protein [7].

In order to check the accuracy of the method, an analysis of proteins with a known amino acid composition was carried out. Egg albumin, chymotrypsinogen and pepsinogen were chosen for this experiment. The method used was analogous to that applied to α-crystallin.

Determination of N-terminal amino acids was carried out by Sanger's method [8]. Fifty mg of protein was dissolved in 2.5 ml of water and 100 mg of DNFB dissolved in 2.5 ml of ethanol was added to the protein solution. Reaction was carried out for 2 hrs at room temperature and with constant stirring. The protein was separated by centrifugation, washed with water, then with ethanol and finally with ether. DNP-protein was hydrolyzed with 6N HCl for 10 hrs at 110°. The hydrolyzate was washed several times with small portions of ether. The ether extract was washed with water and the washings combined with the aqueous fraction. Ether was removed by evaporation, the dry residue dissolved in ethanol and applied to the chromatogram for the identification of the N-terminal amino acid. A mixture of 10% ethanol and 90% benzyl alcohol (1:9) saturated with a phthalate buffer, pH 6, was used as the solvent. The paper was treated with this solution prior to its use. In order to identify amino acids remaining in the aqueous fraction, the latter was evaporated and subjected to chromatographic analysis.

Determination of C-terminal amino acids was carried out by means of the carboxypeptidase method [9]. A carboxypeptidase preparation which had been recrystallized many times was used. The reaction was conducted in the following manner: 20 mg of protein was dissolved in 1 ml of slightly alkaline water (pH 8) containing a small amount of diisopropylfluorophosphate. Upon the addition of 0.1 mg of the enzyme the mixture was incubated at 20°. The reaction was also carried out at 0°. Incubation time was from 2 min to 1 hr. The reaction was stopped by heating the mixture in boiling water for 10 min. After centrifugation the solution was evaporated and investigated on a one-dimensional chromatogram; butanol-acetic acid mixture was used as the solvent.

RESULTS

Figure 1 represents a two-dimensional chromatogram of the DNP-amino acids from the α-crystallin hydrolyzate.

In Levy's method there is an indication about the unsatisfactory separation of dicarboxylic amino acids, leucine and isoleucine. We were not able to obtain a sufficiently good separation of aspartic and glutamic acids

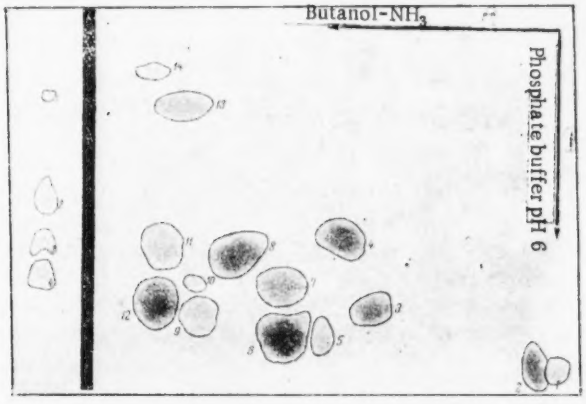

Fig. 1. Two-dimensional chromatogram of the DNP-amino acids from the α-crystallin hydrolyzate (ether fraction): 1) aspartic acid; 2) glutamic acid; 3) serine; 4) glycine; 5) threonine; 6) proline; 7) alanine; 8) dinitrophenol; 9) valine; 10) methionine; 11) phenylalanine; 12) leucine + isoleucine; 13) lysine; 14) tyrosine. On the left-hand side – one-dimensional chromatogram of the aqueous fraction: 1) cysteine; 2) lysine; 3) arginine; 4) histidine.

TABLE 1

Amino Acid Composition of α-Crystallin

Amino acid	Optical density coefficients	No. of amino acid residues in molecule	Weight of amino acid residue in g-moles of protein	Weight of amino acid residue in 100 g of protein	Weight of amino acid in 100 g of protein
Aspartic acid	0.0485	326	37500	4.76	5.51
Glutamic acid	0.1360	914	118 000	15.00	16.94
Threonine	0.0276	185	18 685	2.37	2.80
Serine	0.0613	412	35 844	4.55	5.50
Valine	0.0545	366	36 234	4.60	5.44
Alanine	0.0413	278	19 738	2.65	3.14
Leucine + isoleucine	0.1802	1211	136 843	17.38	20.01
Glycine	0.0547	368	21 000	2.67	3.5
Methionine	0.0134	90	11 800	1.50	1.7
Phenylalanine	0.0793	533	78 351	10.00	11.18
Proline	0.1066	720	69 840	8.87	(10.5)5.2***
Lysine	0.0385	260	33 280	4.23	4.82
Cysteine	0.0033	22	2266	0.29	0.34
Arginine	0.0453	305	47 580	6.04	6.74
Histidine	0.0570	383	52 471	6.66	7.54
Tyrosine	—	350	57 050	7.25	8.05
Tryptophan	—	57	10 602	1.34	1.47
Total	—	6780	787 084	100.16	115.20

* "Molar fractions" according to Levy's terminology [4].

** Molecular weight of α-crystallin 800,000 [4].

*** For explanation see text.

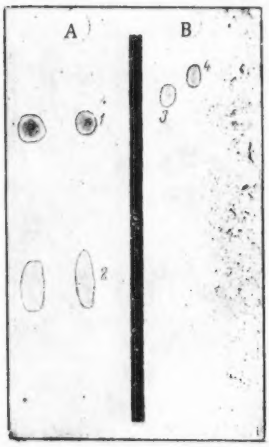

Fig. 2. Chromatograms of the terminal amino acids of α-crystallin. Left-hand side – C-terminal amino acids; right-hand side – N-terminal amino acids. 1) Alanine; 2) leucine; 3) glutamic acid; 4) aspartic acid.

and of the group: methionine, valine, leucine and isoleucine, despite the repeated development of the chromatograms [10]. However, an examination of the chromatograms under ultraviolet light permits a more exact determination of the borders of the closely located colored spots. Because DNP-aspartic acid was slowly extracted with ether, a more prolonged extraction was used; without this modification part of the DNP-aspartic acid remained in solution. In the case of this amino acid the experimental results did not check well, which could probably be explained by the incomplete reaction with DNFB under the conditions of the experiment. DNP-lysine was found both in the ether as well as in the aqueous fractions; the total amount of lysine in both fractions gave close values for different hydrolyzates. Data for the remaining amino acids in parallel hydrolyzates checked satisfactorily. Because of the inaccuracy in the quantitative determination of amino acids by paper chromatography, large numbers of hydrolyzates was analyzed quantitatively.

Table 1 represents the average values of the amino acid content in α-crystallin. Optical densities of the corresponding DNP-amino acids were multiplied by the correction factors determined by Levy [4] during analyses of amino acid mixtures. In the first column of Table 1 optical densities for each amino acid are expressed as optical density coefficients which represent parts of optical density for each amino acid as compared to the total density of the hydrolyzate.

As can be noted from the reported data, α-crystallin is distinguished by quite complete amino acid composition, as 17 different amino acids have been isolated from it.

In the determination of N-terminal amino acids, two glutamic acid residues per molecule of protein were found in the ether fraction. Only DNP-lysine was found in the aqueous fraction.

It was observed during the determination of C-terminal amino acids that even after a brief incubation period (3 min) at room temperature alanine, glycine, tyrosine, valine, phenylalanine and leucine were split off. In order to retard the reaction, incubation was carried out at 0° using the same amounts of the protein and the enzyme. In this case upon a 2-min incubation period alanine and leucine were found in the solution, but on the chromatograms the alanine spot had a greater color intensity than the spot of leucine.

Chromatograms of C- and N-terminal amino acids are shown in Fig. 2.

DISCUSSION OF RESULTS

The method of amino acid determination as their DNP-derivatives was used successfully in the analysis of some proteins [11, 12]. As in other methods of amino acid determinations which employ paper chromatography, this method cannot be considered strictly quantitative. In this case losses due to incomplete reaction between DNFB and the amino acids, due to adsorption of DNP-amino acids on paper, etc. cannot be avoided. However, these errors have the same magnitude as demonstrated by good correlations between parallel determinations, if

TABLE 2

Amino Acid Composition of Egg Albumin, Chymotrypsinogen and Pepsinogen Determined by Means of the Dinitrophenyl Method

Amino acid	Egg albumin		Chymotrypsinogen		Pepsinogen	
	amino acid content in %					
	by means of DNP-method	reported in literature [13]	by means of DNP-method	reported in literature [13]	by means of DNP-method	reported in literature [13]
Aspartic acid	27	26	20.1	18.3	23.96	25.7
Glutamic acid				10.9		
Serine	6.84	8.15	8.5		10.45	13.4
Glycine	2.02	3.05	6.07	6.3	6.6	6.5
Threonine	3.5	4.0	8.0	10.7	6.3	7.7
Proline	8.0	3.6	8.9	4.0	10.3	5.6
Alanine	7.8	6.7	7.0	5.8	4.3	5.6
Valine	6.2	7.0	11.5	10.3	7.3	7.6
Lysine	3.3	6.3	5.1	7.7	2.36	4.2
Phenylalanine	7.0	7.6	4.5	3.77	8.4	8.0
Leucine + isoleucine	19.8	16.2	17.0	14.0	19.67	20.0
Cysteine	1.35	1.35	1.45	1.1	0.56	1.1
Arginine	8.5	8.0	2.7	2.7	2.8	1.3
Histidine			1.8	1.16	1.45	1.4
Methionine	6.0	5.2	—	—	—	—
Tyrosine*	4.0	3.68	—	—	—	—
Tryptophan	1.6	1.20	—	—	—	—

* Tyrosine and tryptophan in egg albumin were determined spectrophotometrically.

the standards are strictly adhered to at each step of the determination. This permits one to introduce corresponding corrections in the determinations and, therefore, obtain quantitative results. The calculation method is also helpful. The value of the method is also increased by the fact that only 10-15 mg of material and 1-2 weeks of work are needed for the complete determination of amino acid composition of a protein. Another point in favor of this method is the yellow color of DNP-amino acids which eliminates the necessity of a special color test for the detection of the spots.

It was pointed out previously in this report that determinations of amino acid composition of egg albumin, chymotrypsinogen and pepsinogen were carried out in order to evaluate the experimental method (Table 2).

As can be seen from Table 2, data obtained by the dinitrophenyl method correspond closely to those reported in the literature for the majority of amino acids; deviations exist for some amino acids; they are particularly large in the case of proline. Increased results for proline, leucine – isoleucine and alanine can be explained by the close location on paper of these amino acids and dinitrophenol which is a decomposition product in the reaction between protein and DNFB. Increase in proline content for the three proteins gives close value and represents on the average 110%. Apparently it seems possible to avoid increased values for the above amino acids by removing excess dinitrophenol from the solutions prior to their application to the chromatogram.

Decreased lysine content in the investigated proteins as compared to the reports in the literature, apparently can be explained by an incomplete reaction between that amino acid and DNFB.

In calculating the contents of serine and threonine a correction equal to 11 and 5%, respectively, was introduced. It was done on the basis of indications in the literature that partial decomposition of these amino acids takes place upon hydrolysis [13]. Despite the introduced corrections, decreased values were obtained for these amino acids. Reasons for this phenomenon are not clear.

On the basis of the data obtained for the analyses of egg albumin, chymotrypsinogen and pepsinogen, it was considered possible to introduce a correction for the proline content of α-crystallin and thus decrease the obtained value. In Table 1 next to the recalculated values for proline, the experimental data are given in parentheses.

SUMMARY

Amino acid composition of α-crystallin from the lenses of cattle was determined by means of the dinitrophenyl method. α-Crystallin has the following composition (in %): aspartic acid – 5.5; glutamic acid – 17; threonine – 2.8; serine – 5.5; valine – 5.4; alanine – 3.1; leucine + isoleucine – 20; glycine – 3.5; methionine – 1.7; phenylalanine – 11.2; proline – 5.2; lysine – 4.8; cysteine – 0.3; arginine – 6.7; histidine – 7.5; tyrosine – 7.4; tryptophan – 1.3.

Terminal amino acids in α-crystallin were also determined: N-terminal amino acid is glutamic acid (2 residues per molecule of protein), C-terminal amino acid is alanine.

The author wishes to express her gratitude to the Active Member of the Academy of Medical Sciences USSR, Professor V. N. Orekhovich for his guidance in carrying out his work.

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INVESTIGATION OF GLYCININE FRACTIONS IN THE ULTRACENTRIFUGE

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It was demonstrated by means of an ultracentrifuge in a previous investigation [1] that glycinine from soy bean cotyledons dissolved in 10% NaCl is composed of two independently sedimenting components having sedimentation constants (S) equal to 14.0 and 8.1 Svedberg units. The molecular weight of those components was 330,000 and 126,000, respectively. It was also demonstrated that upon dissolving the protein in a salt-free solution of cysteine, a third component appeared which had S = 10.7 and molecular weight equal to 245,000. Existence of this component depended upon the presence of some very labile compounds because upon the addition of 10% NaCl to the solution the protein transformed to its original composition.

Naismith [2] also pointed out the possibility of a reversible association of glycinine components at low ionic strengths.

The purpose of this investigation was to isolate components S_8 and S_{14} in order to conduct further experiments with them.

Fractionation of Glycinine

The same preparation of glycinine from soy bean cotyledons as that employed in the previous investigation [1] was used in this work.

The whole fractionation procedure was carried out in the cold. Glycinine was dissolved in 10% NaCl and filtered, then a saturated $(NH_4)_2SO_4$ solution was added until saturation reached 70%. Precipitated protein was separated by centrifugation, the precipitate dissolved in 10% NaCl and dialyzed against distilled water. After the dialysis was completed, the protein was dried by lyophilization. This preparation referred to as "total"protein was subjected to further fractionation by means of variable saturations with ammonium sulfate.

Each fraction was again dissolved in 10% NaCl, dialyzed and dried by lyophilization.

When the separate fractions are described in this article, they are designated by the range of the corresponding ammonium sulfate concentrations; for example, "fraction 50-60" denotes fraction isolated in the range of 50-60% saturation of the protein solution with ammonium sulfate, etc.

During centrifugation of protein precipitated at 65-70% saturation with ammonium sulfate, part of the protein remained suspended in solution and settled down upon standing in the cold for 24 hrs. Protein fraction obtained in this manner is referred to as 65-70 (II).

Experiments with the Ultracentrifuge

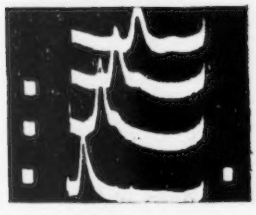
The experiments were carried out under the same conditions as those described in the previous report [1].

It can be noted from Table 1 that in single cases, traces of the heavier component S_{20} appeared together with components S_8 and S_{14}. Since component S_{20} was not present in detectable quantities in the "total" protein, it was, therefore, presumed to be an association product of component S_{14}.

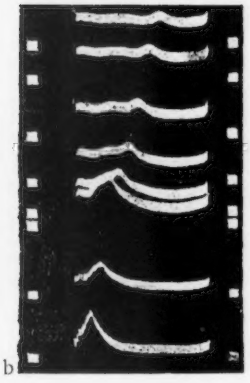
TABLE 1

Characteristics of Sedimentation Diagrams of Separate Globulin Fractions from Soy Bean Cotyledons

Fraction (% saturation $(NH_4)_2SO_4$)	Solvent	Protein conc. in %	No. of components	S		
				1	2	3
70 ("total")	10% NaCl	1.0	2	8.46	14.36	—
55—60	"	1.0	1	—	13.74	—
65—70	"	1.0	1	—	14.55	traces
65—70	"	0.7	1	traces	13.65	—
65—70	H_2O, cysteine (10 mg in 1 ml)	1.0	2	7.95	13.65	—
65—70	H_2O, cysteine (1%); after dissolving the protein enough NaCl was added to yield a 5% solution	0.5	1	—	14.12	—
65—70 (II)	10% NaCl	1.0	2	7.16	13.08	—
65—70 (II)	H_2O, ATP (1%)	1.0	1	traces	13.0	traces
65—70 (II)	H_2O, ATP (1%), after dissolving the protein enough NaCl was added to yield a 5% solution	0.5	1	—	13.08	—



a



b

Fig. 1. Sedimentation diagram of fraction 65-70: a) protein concentration — 1%; b) 0.7%.

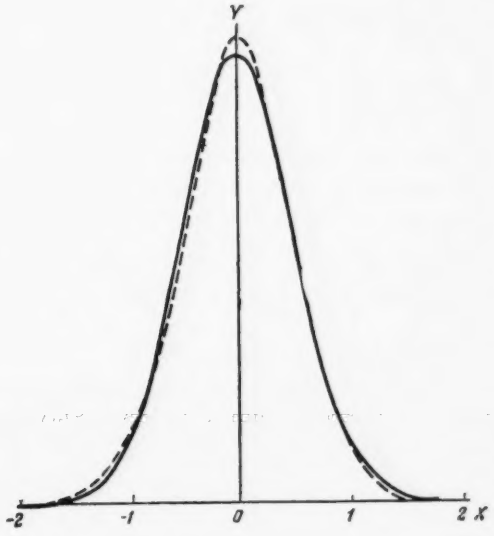


Fig. 2. Diffusion curve of fraction 65-70: continuous line — ideal curve, dotted line — experimental curve.

Sedimentation diagram for "pure" component S_{14} is presented in Fig. 1.

Diffusion coefficient was also determined for this component; it appeared that $D_{2m} = 3.57 \pm 0.18$, $D_A = 3.07 \pm 0.01$. The normal diffusion diagram is shown in Fig. 2.

As is evident from the examination of the sedimentation diagram (Fig. 1), this fraction contains about 10% of the heavy S_{20}-component. Because coefficient D_A is particularly sensitive to the heavy components, in this case coefficient D_{2m} should be employed for the calculation of M according to Svedberg's equation, especially since the low-molecular weight admixture is absent as can be noted from the normal curve. Substituting S = 14

and D = 3.57 into Svedberg's equation value M = 380,000 is obtained which is in good agreement with the preliminary estimation. If one considers this corrected value of M, then it appears that the molecular weights of S_8-, S_{11}-, S_{14}-components are in the approximate ratios of 1:2:3.

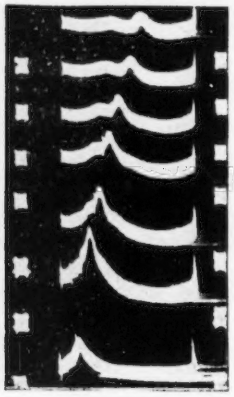


Fig. 3. Sedimentation diagram of fraction 65-70 (II).

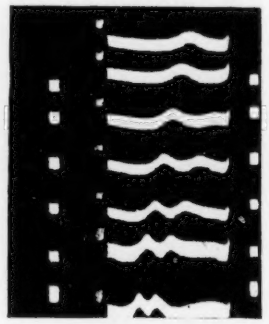


Fig. 4. Sedimentation of fraction 65-70 dissolved in the presence of cysteine.

Upon dissolving in 10% NaCl, fraction 55-60 consists practically of a single component having S = 13.74.

As can be noted from Fig. 1, a, fraction 65-70 contains only traces of component S_{20} while S_8-component is absent, When this fraction is diluted, S_{20}-component disappears almost completely, but traces of S_8-component show up (Fig. 1, b).

This dependence of the sedimentation behavior upon the protein concentration is typical for the equilibrium between a monomer and an oligomer; with increased dilution this equilibrium should shift toward the monomer – in this case S_8-component.

Fraction 65-70 (II) contains both components (Fig. 3); their S is somewhat lowered, probably the result of increased asymmetry of globules due to possible modifications after repeated drying or reprecipitation.

Experiments with Cysteine

In the previous work dissolution of the protein in water in the presence of cysteine at pH ≈ 7 led to the appearance of $S_{10,7}$-component, however the addition of salt caused disappearance of the supplementary component.

Experiments on the influence of cysteine were carried out on component S_{14}. The experimental design was somewhat altered: 1 ml of water and 10 mg of cysteine were added to 10 mg of the protein. As can be noted from Fig. 4, under these conditions, i. e., in the presence of cysteine the light component S_8 appears in place of component S_{14}.

It is extremely important that as in the experiments with the original glycinine preparation the addition of salt led to the disappearance of the component which was formed under the influence of cysteine.

Therefore, the results of previously described experiments carried out on the original glycinine preparation as well as the results obtained in experiments investigating the influence of cysteine on S_{14}-component of glycinine, fully support the fact that cysteine decreases and 10% NaCl solution increases the number of components present in the protein solution. In addition, the reported experiments point out that the action of cysteine is reversible and eliminated by the addition of salt.

Experiments with ATP

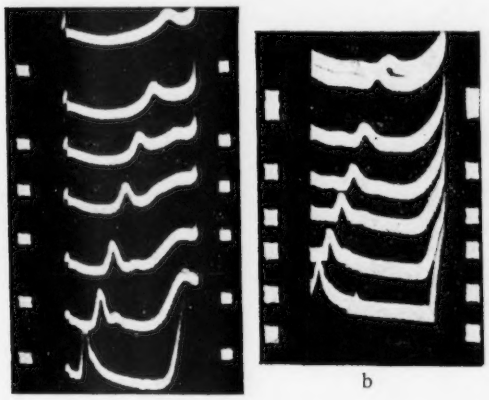
Experiments designed to explain the action of ATP on glycinine from soy bean cotyledons and on edestin from hemp seed were carried out in the following manner.

ATP solution* neutralized with $NaHCO_3$ was added to the protein dispersed in double-distilled water. The mixture was left standing at 37° for 1-1.5 hrs, then the undissolved protein was removed by centrifugation. Samples of the clear solution were taken for the determination of total nitrogen; after precipitating the dissolved protein according to Barnstein's method, samples were taken for the determination of nonprotein nitrogen.

TABLE 2

Solubility of Glycinine and Edestin in the Presence of ATP

Conditions of dissolution	Time of dissolution (hrs)	Amount of dissolved protein in % of initial quantity
pH 6		
Glycinine 30 mg + 7 ml water	1.5	0
Glycinine 30 mg + 7 ml water + 10 mg neutralized ATP	1.5	46.2
pH 6.98		
Glycinine 30 mg +7 ml water	1.0	0.0
Glycinine 30 mg + 7 ml water + 3 mg neutralized ATP	1.0	83.0
Edestin 30 mg + 7 ml water	1.0	0.0
Edestin 30 mg + 7 ml water + 3 mg neutralized ATP	1.0	14.8



a b

Fig. 5. Sedimentation diagram of fraction 65-70 (II): a) in the presence of ATP; b) the same, upon addition of NaCl.

The quantity of the dissolved protein was calculated on the basis of the obtained data. Corresponding values are presented in Table 2.

As can be seen from the data in Table 2, ATP has solubilizing action on proteins.

Similar solubilizing action of ATP on reserve proteins of seeds could play an important part in the life processes of an organism, specifically during germination of seeds by promoting the transformation of insoluble proteins into a soluble condition Neuberg and co-workers [3, 4] also pointed out the solubilizing action of ATP salts and hypothesized that the wide distribution of ATP in all cells of the organism indicates its role in solubilizing of insoluble matter and its transport in the organism.

Experiments carried out by means of ultracentrifuge showed that the dissolution of fraction 65-70 (II) in the presence of ATP (10 mg of protein + 10 mg of ATP + 1 ml of water, pH ≈ 7) leads to almost complete association and disappearance of the S_8-component on the sedimentation diagram and to partial association of the S_{14}-component causing the appearance of traces of S_{20}-component (Fig. 5, a). Addition of NaCl practically destroys the S_{20}-component but does not touch the S_{14}-component (Fig. 5, b).

* ATP preparation, Ivanov Meat Combine.

Thus during the action of ATP on the protein a transformation of the latter takes place, which is accompanied by the association of the lighter component. The action is quite stable and is not eliminated by the addition of NaCl as in the case of the action of cysteine on the protein.

Since in all of the experiments proteins were transferred from one solvent into another one, the observed phenomena cannot be explained on the basis of different solubilities of "individual" components previously present in the fractions. Consequently, the redistribution of the components is influenced by the presence of true equilibria between monomer and oligomer forms of the same protein.

SUMMARY

Depending upon the conditions of dissolution "individual components" of glycinine from soy bean cotyledons are capable of transformation into one another; i. e., are oligomers of the same basic submolecule – probably the S_8-component with a molecular weight of the order 128,000. During association the trimer (i. e., the S_{14}-component) seems to be most stable. Apparently this condition is influenced by some energetic and structural factors which are not clear at present.

Probably, depending upon the conditions of dissolution, an equilibrium is slowly established during which separation into individual components takes place. Polyvalent ions of the cysteine and ATP-type considerably modify this equilibrium. Solubilizing action of cysteine on the protein is demonstrated by the increase in the number of protein components present in solution.

The possible mechanism of the action of polyvalent ions may be due to their being tied up with the protein macromolecule, and thus changing its effective charge and modifying the electrostatic forces among the globules which in turn influence the equilibrium between monomer and oligomer forms. Experiments should be carried out in order to check this hypothesis.

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THE USE OF USSR-MADE ION-EXCHANGE RESINS IN THE ISOLATION OF NUCLEOTIDES FROM PANCREAS HYDROLYZATES

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The isolation of large quantities of pure nucleotides from tissue hydrolyzates presents a difficult task. The application of synthetic ion-exchange resins provides a comparatively easy means for the isolation of the above-mentioned compounds, and has become in recent years the most effective method for the analysis of nucleic acids and their derivatives [1-3].

The pronounced anionic character of the nucleotides determines their behavior during ion-exchange and allows their separation from ribosides, free bases and other compounds present in the complex biological mixture.

The present communication deals with the use of USSR-produced ion-exchange resins in the isolation and purification of mononucleotides obtained from the alkaline hydrolysis of nucleoprotein from the pancreas of large cattle.

The resins investigated in the present work are known under the names NO, MMG-1, EDE-10 and AN-2F. Preliminary experiments with the resins NO and MMG-1 showed that, although phosphorus-containing compounds were easily adsorbed by them, the subsequent elution with hydrochloric acid caused partial dissolution of the resin itself and the appearance in the eluate of excess nitrogen, as estimated by the Kjeldahl method. Therefore, all subsequent experiments were carried out with the resins AN-2F and EDE-10, which were used for the isolation of nucleotides. The resins were used as a free base, and also in the chloride, carbonate, and acetate forms.

The acetate form of the resin was found most convenient for the maintainance of a steady pH during the adsorption of the nucleotides onto the columns. However, determinations of resin capacity, using mixtures of crystalline nucleotides, showed that optimal conditions of adsorption could not be reached using the exchanger in one salt form only.

The following amounts of nucleotide mixtures were taken up from a neutral solution by the various resins after 1 g of each resin (acetate) was shaken in a shaker for 3 hours:

MMG-1 – 3.3 mg phosphorus, or 43.4 mg of the nucleotide mixture.

AN-2F
EDE-10 } – 3.5 mg phosphorus, or 46.0 mg nucleotide mixture.

NO – 5.0 mg phosphorus, or 65.8 mg nucleotide mixture.

Thus all the resin makes quoted above showed an approximately equal and low capacity. When the adsorption was carried out from a nucleotide solution acidified to 1-2% with acetic acid, the capacity of the resins AN-2F and EDE-10 increased 10-15-fold. The capacity could also be increased if the resin on the column was pretreated with a 5% acetic acid solution.

When adsorption was carried out directly from an acidified hydrolyzate of a pancreas extract, where the process was complicated by the presence of a large amount of salt, protein and amino acids, the uptake of phosphorus-containing compound was incomplete and represented but 60-70% of the total hydrolyzate phosphorus. Under those conditions 1 g of the resin AN-2F took up 10-20 mg phosphorus, or 100-250 mg of nucleotides.

The fraction adsorbed could be increased to 80-97%, and the absolute amount of compounds adsorbed by 1 g of resin could be brought to 30-40 mg P, or 300-400 mg of nucleotides, if the pancreatic nucleoprotein hydrolyzate was treated, before adsorption, with the cation-exchange resin KU-2, and the protein which subsequently precipitated on acidification was rejected.

The adsorbed nucleotides were eluted with cold 0.05 N HCl. In the case of large-scale preparations destruction of the nucleotides by prolonged action of the acid was prevented by enclosing the column in a jacket through which ice-cold water was passed.

Nucleotides appeared in the eluate as soon as it began to issue, or after one blank fraction had passed out. The elution was usually completed between the point when the eluate showed a faintly acid reaction and the time when its pH reached that of the eluant.

When the resin AN-2F was not loaded to capacity, nucleotide elution commenced after a prolonged blank fraction, and, once started, proceeded rapidly and quantitatively. In the case when the amount of nucleotide adsorbed per 1 g of resin was equivalent to 40-50mg phosphorus, the desorption of nucleotides by 0.05 N HCl was prolonged and did not always reach completion.

This high retentivity of the resin for nucleotides at its maximal capacity was probably due to the dissimilarity of the active centers of the resin AN-2F and EDE-10.

The large capacity for nucleotides and the chemical and physical stability of the resins AN-2F and EDE-10 allow their use for the quanitative isolation of the mixed nucleotides and their subsequent fractionation by chemical or chromatographic means.

A fractionation procedure for the treatment of the HCl eluate containing the mixed nucleotides is given below.

The acid eluates were neutralized with 4% NaOH as soon as they were collected. The individual fractions were combined and the solutions evaporated. To the evaporated solutions was added a saturated solution of $Ba(OH)_2$ to pH 8-9, and two volumes of alcohol. A flocculent precipitate of the barium salts appeared after standing in the ice-chest. The barium salts of the nucleotides were then separated from the inorganic phosphate by virtue of the solubility of the former in water.

The inorganic phosphate contamination of the mononucleotide fraction of the nucleoprotein hydrolyzate comprised about 35-50% of the totally eluted phosphorus. Preliminary removal of the inorganic phosphate gave higher subsequent yields of mononucleotide from the columns.

Free nucleotides were obtained from the barium salts by allowing their solution to pass through small columns containing the cation-exchanger KU-2 in the hydrogen form. The eluates from this column were evaporated to a small volume and the mononucleotides precipitated with 10-20 volumes of alcohol. After standing in the ice-chest the precipitate was separated, washed and dried in the air. The preparation of mixed mononucleotides thus obtained gave a strong pentose reaction, burned with strong effervescence leaving no mineral residue and was ninhydrin- and biuret-negative.

The various nucleotides in the mixtures were identified by paper chromatography using the solvent isobutyric acid–0.5 N ammonia (10:6) [4]. Adenylic and guanylic acid preparations were employed as markers. The Brumberg ultrachemiscope [5] was used for the detection of the spots on the chromatograms. The following distribution coefficients were obtained using Leningrad-2 chromatographic paper:

uridylic acid guanylic acid	0.30	cytidylic acid	0.51
		adenylic acid	0.65

Paper chromatograms were also developed by the method recommended by Reindel and Hoppe [6] for amino acids, peptides and proteins. After the appropriate treatment the mononucleotides appeared on the paper as dark blue spots.

The nucleotide mixture in hand was easily and completely fractionated by the method of Laufer and Charney [7] into two fractions – purine and pyrimidine nucleotides – which greatly facilitated the subsequent isolation of the individual nucleotides in pure form and in quantities sufficient for further work.

The process of ion exchange on the columns and that of subsequent isolation of the individual nucleotides was checked by analysis for phosphorus and nitrogen (the latter not in all cases). The phosphorus was determined according to Fiske and Subbarow and the nitrogen by a microadaptation of the Kjeldahl procedure.

EXPERIMENTAL

Column preparation. The samples of the various ion-exchange resins, mesh size 0.25-0.50 mm, were stirred in a beaker with 2N HCl and allowed to stand overnight. The resin was filtered off on a Büchner funnel and washed with water. Treatment with hydrochloric acid was then continued until a positive reaction for iron was no longer obtained. The resin was then washed with warm distilled water until the washes were neutral; this was followed by stirring for 2-3 hrs with 2% sodium hydroxide solution.

When the (OH) form of the resin was desired, it was washed after the alkaline treatment with boiled distilled water until the washes were free from chloride. In order to obtain the acetate form, the resin was washed with water to neutral reaction in the washings, treated with 5% acetic acid and transferred to a column. The column – a glass tube 35-50 cm long and 0.75-1.25 cm in diameter – was filled with the resin and connected to the reservoir containing the solution under investigation.

TABLE 1

Separation of Nucleotides on a Column with the Resin AN-2F

Fraction	Volume of sol'n in ml	P content, mg	N/P ratio
elution with 0.05 *N* HCl			
1 (before appearance of weak acidity)	400	120	—
2	1100	687	2.5 : 1.0
3	300	75	—
elution with 0.1 *N* HCl			
4	300	77	—
5	300	65	—
elution with 0.5 *N* NaOH			
6	500	270	—
Totally eluted		1294 at	98.7%

Preparation of nucleotide mixtures. The nucleotides were prepared from the pressings remaining after the extraction from the pancreas of the hypertensive hormone by the method of Andreev [8]. The semifluid mass of the pancreatic pressings was extracted with 10 volumes of distilled water, with heating, over 4-5 hrs. The aqueous extracts were concentrated by evaporation and precipitated with two volumes of alcohol. After standing in the cold the precipitate was separated, washed with alcohol and dried in the air.

Sixty g of the nucleoprotein was hydrolyzed with 10 times its volume of N NaOH for 24 hrs at 37°. The precipitated pyrophosphate was separated and rejected. The solution was diluted 4-5-fold with distilled water and the cation-exchanger KU-2, in the H-form, was stirred in. After removal of the cation and of the protein which precipitated on subsequent acidification, the hydrolyzate contained 8.1 g of nitrogen and 1.5 g of phosphorus. The N/P ratio was 5.4 : 1. No loss of phosphorus occurred during the treatment with the resin KU-2.

Isolation of nucleotides using the resin AN-2F. A column with dimensions 65 cm × 1.12 cm^2 was loaded with 25 g of the resin in the acetate form and 200 ml of 5% acetic acid was allowed to pass through. The hydrolyzate was then allowed to pass through the column with a flow rate 0.5-0.7 ml/min. The column adsorbed 1.31 g of phosphorus, that is, 88% of the starting material.

The nucleotides were eluted with cold, 0.05 N HCl, followed in the end by 0.5 N NaOH. The flow rate was 0.6 ml/min.

The experimental results are presented in Table 1.

The neutralized acid eluates were combined and evaporated to a small volume. After the precipitation of the barium salts and the removal of the inorganic phosphate residue the final solution of the nucleotide mixture contained 464 mg nucleotides, or 45.3% of the total phosphorus desorbed from the column.

The solution of the barium salts of the nucleotides was passed through the cation-exchanger KU-2 in the H-form. The eluate was evaporated in vacuo, at a temperature not exceeding 40°, to a very small volume and was then poured into cold alcohol. This resulted in the precipitation of a heavy viscous mass which changed into a crystalline precipitate on rubbing. The precipitate was allowed to stand in the ice-chest and was finally separated and dried in the air. The yield of the nucleotide mixture was 6.7 g. The preparation was free from inorganic con-

tamination, it was biuret and ninhydrin-negative and contained 20.1% nitrogen and 7.6% phosphorus. Paper chromatography revealed the presence of four UV-absorbing spots with R_f values 0.27, 0.32, 0.55 and 0.68, respectively.

The isolation of nucleotides from an aqueous pancreas extract using the resin AN-2F. Three hundred g of dried, fat free, finely powdered pancreas was stirred mechanically into boiling distilled water. Boiling was continued for 3-4 hrs. The insoluble residue was separated and re-extracted likewise with boiling water. The combined aqueous extracts contained 1.17 g phosphorus.

The nucleoprotein precipitation step was bypassed and the evaporated aqueous extract was adjusted with 40% NaOH to 1 N alkali and was hydrolyzed for 24 hrs at 37°. After removal of the precipitate which appeared, the hydrolyzate was diluted with water to 750 ml.

TABLE 2

Separation of Nucleotides with the Resin AN-2F

Fraction	Volume of sol'n in ml	P content in mg
eluted with 0.05 *N* HCl		
1 (blank frac.)	700	0
2	190	190
3	200	300
4	100	10
Totally eluted:		500. at 100%

Five hundred ml of the hydrolyzate, containing 700 mg phosphorus, was acidified with acetic acid and left in the ice-chest overnight. A small protein precipitate formed and was separated and rejected. The column (50 cm × 1.12 cm^2) of the exchanger AN-2F in the acetate form was washed with 5% acetic acid immediately before passing through the nucleotide solution. The flow rate for the nucleotide solution was 0.6 ml/min. The filtrate contained 200 mg phosphorus. The column adsorbed 500 mg of phosphorus (71.4%). The nucleotides were eluted with cold 0.5 N HCl.

The results are presented in Table 2.

After the precipitation of the barium salts and the removal of inorganic phosphate the mononucleotide solution contained 152 mg P, that is, 30% of the total phosphorus removed from the column.

Isolation of the nucleotides using the resin EDE-10. A column of dimensions 27 cm × 0.38 cm^2 was charged with 6 g of the resin prepared in the acetate form; the column was washed with 5% acetic acid.

The nucleotide solution, containing 100 mg nucleotide phosphorus in 150 ml was acidified slightly and allowed to pass through the column at a rate 0.5-0.7 ml/min. Because of the light yellow color acquired by the resin as a result of multicycle regeneration, it was possible to ascertain visually that the upper 17-18 cm of the column was occupied by the adsorbed nucleotides.

Elution was carried out with cold 0.05 N HCl. The first 110 ml of the eluate contained no nucleotides. Eluate collection was commenced when the adsorbed substance was seen to have reached the bottom of the column. One hundred mg of mononucleotide phosphorus was eluted. The yield in the elution was thus 100%.

Chromatographic separation. In a series of experiments chromatographic separation of the nucleotides was attempted by elution from the column with dilute acid of gradually increasing concentration. The sequence of nucleotide appearance from the column was checked by paper chromatography. The first nucleotide eluted with 0.1% formic acid was cytidylic acid. Paper chromatography showed one spot with an R_f corresponding to that of cytidylic acid (0.52). When the formic acid concentration in the eluant was increased, adenylic acid, as well as cytidylic, was eluted. Paper chromatography revealed the presence of a new spot, with an R_f value of 0.68. When elution with 1% formic acid was continued, guanylic acid appeared and correspondingly a third spot, R_f = 0.3, made its appearance on the chromatogram. In order to elute this nucleotide completely off the column the hydrochloric acid concentration had to be increased to 1.0 N.

The chromatographic separation of nucleotides was carried out with the resin AN-2F loaded to capacity; under other conditions guanylic acid was eluted completely with 0.05-0.1 N HCl.

Separation of mononucleotides. The crystalline mixture of mononucleotides was separated into purine and pyrimidine nucleotides.

Following the conditions postulated by Laufer and Charney [7], the copper salts of the nucleotides were separated into two fractions:

Scheme for the chromatography of nucleotides separated according to the method of Laufer and Charney [7]: Solvent: isobutyric acid – 0.5 N ammonia (10 : 6). I) Mononucleotide mixture: 1) guanylic acid and uridylic acids; 2) cytidylic acid; 3) adenylic acid; II) pyrimidine nucleotides: 1) uridylic acid; 2) cytidylic acid; III) purine nucleotides: 1) guanylic acid; 2) adenylic acid.

1) the water-insoluble copper salts of the purine nucleotides;

2) the water-soluble copper salts of the pyrimidine nucleotides.

The separation proceeded without difficulty. The completeness of the separation is evident in the paper chromatogram (see figure).

Isolation of guanylic acid. The nucleotide fraction which was eluted from the AN-2F column by 0.5 N NaOH solution (270 mg P), and which contained mainly guanylic acid, was treated separately. Precipitation of the barium salts resulted in the removal of a small precipitate containing inorganic phosphate in the form of $Ba_3(PO_4)_2$. After removal of the barium by the cation-exchanger KU-2, H-form, the nucleotides were precipitated by the addition of alcohol. In order to free the guanylic acid from pyrimidine nucleotide contamination, the preparation was dissolved in water, the solution adjusted to pH 7-8 and brought to boil; the reagent consisting of 10 ml of $CuSO_4 \cdot 5H_2O$ solution (25 g in 100 ml water) and 2.5 ml of $NaHSO_4$ solution (28 g in 100 ml water) was then added to the boiling solution. Heating was continued for 1-2 min and the solution was allowed to cool to room temperature. The precipitated copper salts of the purine nucleotides were collected by centrifugation, washed with water and decomposed with hydrogen sulfide. After removal of the CuS precipitate the filtrate was adjusted to pH 7-7.5 by the addition of concentrated ammonia and the nucleotides were precipitated with two volumes of alcohol. The suspension was allowed to stand in the ice-chest overnight, the precipitated ammonium salt of guanylic acid was collected and again reprecipitated from water with an equal volume of ethanol.

The ammonium salt of the guanylic acid was converted to the free acid by letting the solution pass through a column charged with the cation-exchanger KU-2, H-form. The eluate was then evaporated to a small volume and poured into cold alcohol. A fine, snow-white precipitate of guanylic acid was obtained. The yield was 1.2 g.

The following analytical results (formula: $C_{10}H_{14}N_5O_8P \cdot 2H_2O$) were obtained:

	Calculated, %	Found, %
Nitrogen	17.6	17.6
Phosphorus	7.8	7.8

Paper chromatography, using the isobutyric acid – 0.5 N ammonia (10 : 6) solvent, revealed the presence of only one spot, corresponding in its R_f value to guanylic acid.

SUMMARY

A method has been developed for the isolation of mononucleotides from alkaline hydrolyzates of either aqueous pancreas extracts or whole pancreas nucleoprotein, using USSR-produced anion-exhcnage resins AN-2F and EDE-10.

The applicability of the cation-exchange resin KU-2 in the H-form for the conversion of the ammonium, sodium and barium salts of nucleotides into the free acids has been demonstrated; no losses of phosphorus were found to occur during the conversion.

It was shown that the combined application of ion-exchange technique and the classical chemical methods permitted the isolation of guanylic acid in a chemically pure form, by a simple and nonlaborious procedure.

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EFFECT OF YEAST EXTRACTS ON IRRADIATED ORGANISMS

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In a previous paper [1] it was shown that spleen regeneration after sublethal irradiation of mice by Co^{60} γ-rays is accompanied by a marked increase in its content of ribonucleic acid (RNA). This result was hypothetically explained on the basis that RNA plays a role in reducing the damage brought about by irradiation, since it is well known that RNA takes part in regenerative processes after the action of other harmful agents [2].

On this basis it might be anticipated that RNA introduction into the irradiated organism might reduce the harmful effects of irradiation. This possibility appeared to be particularly worthy of consideration, since most of the known anti-radiation remedies studied up to this time are ineffective when used after irradiation [3].

The present paper describes studies on the effect of RNA and yeast extracts rich in RNA on irradiated animal and plant organisms.

METHODS

Mice of various strains, white rats, and pea plants served as the test materials. The rats were irradiated with x-rays (170 kv, 15 ma, 0.5 mm Cu + 2.0 mm Al filter, dosage strength 12.5 r/min). The mice and peas were irradiated with Co^{60} γ-rays at a dosage magnitude of 10 to 50 r/min. The mortality rate was measured in the animals, and in the peas, the rate of growth and the precent of abnormal mitoses in the root meristem. Details concerning the technique of irradiation and the radiobiological characteristics of the test materials were fully described previously [4].

To obtain the extracts, the yeast was suspended in a fivefold volume of water, heated to 95° and left at this temperature for 15 min. The suspension was then centrifuged and filtered until there was obtained a transparent, slightly opalescent amber colored liquid, possessing a greenish fluorescence. These extracts were used either directly after preparation (the liquids did not hold up under protracted storage, in view of development in them of abundant bacterial flora) or they were preserved in the form of a dry powder. For irradiation an extract of the dry material was concentrated under vacuum and the brown "syrup" was dried in a vacuum desiccator over sulfuric acid. A solution of these substances has all the properties of the fresh extract.

Nucleic acids were obtained from the yeast by treatment with NaOH and subsequent precipitation of the protein by 40% acetic acid [5]. The RNA was precipitated by alcohol in acid medium and washed with alcohol, acetone and ether.

The extracts were introduced into mice intraperitoneally, several times (immediately after irradiation, after 1 day, and after 2 days), or just once.

The pea sprouts were irradiated 1 day after wetting of the seeds in water and 2 days germination on wet sand, and immediately after irradiation were mixed with a solution of the extract in which the pea grew until the end of the test. For cytological analysis the sprout was fixed 48 hrs after irradiation in a mixture of absolute alcohol, glacial acetic acid and chloroform (6:3:1) and dyed with lacmoid or by the Feulgen method.

For phosphorus determination the material was ashed with HNO_3 and perhydrol. The phosphorus was precipitated in an aliquot by ammonium molybdate, dissolved in ammonia, and reprecipitated with magnesia mix-

ture. The $Mg_2P_2O_7$ obtained, after filtration, washing, and heating, was weighed. Protein determination was carried out by standard methods [5].

All the results were subjected to statistical analysis. The χ^2 method was the chief criterion, and in the case of some of the variants – the Fischer method was used [6].

RESULTS

Anti-Irradiation Effects of Yeast Extracts

The first tests with introduction of RNA into irradiated rats did not give the expected results. In these tests RNA was introduced once, in a quantity of 300 mg/kg after irradiation with a 700 r dosage. All the animals died after 20 days, both in the test animals and controls; the average length of life was 5.64 days in the test animals, and 6.83 days in the controls. The unsuccessful tests may be explained on the basis of an insufficient anti-irradiation effect manifested by RNA alone, and the necessary presence of some supplementary factors. Accordingly, it was decided to set up some tests with yeast extracts rich in nucleic acids.

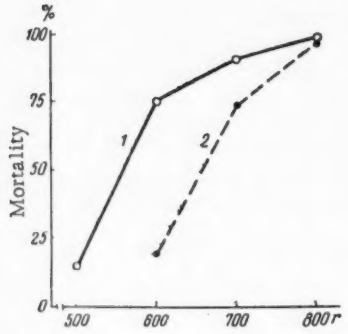

Fig. 1. Relationship of mortality of strain X mice (to 20 days) to γ-ray dosage: 1) control, 2) introduction of 0.3 ml yeast extract after irradiation.

A number of tests were set up with yeast extracts, the general results of which were as follows. Some yeast extracts being used after irradiation noticeably decreased the radiation disease.

In Fig. 1 are presented curves of the relationship of the mortality rate of mice on the irradiation dosage in the controls and triple introduction of 0.1 ml yeast extract.

Figure 1 indicates that yeast extract lowers the irradiation effect approximately by 100 r: a 600 r dosage with the extract gives the same effect as 500 r in the control. The optimum yeast extract dosage is 0.3 ml in an animal weighing 20 g.

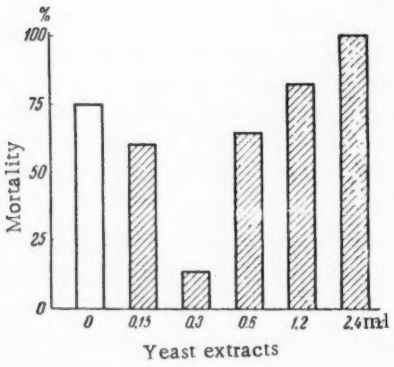

Fig. 2. Effect of introduction of various quantities of yeast extract on mortality of strain X mice, irradiated with γ-rays (dosage 600 r).

From Fig. 2, in which are presented results of the effect on mouse mortality of various dosages of yeast extract, it can be seen that 0.15 and 0.6 ml have a lesser effect and 2.4 ml even increases the animals' mortality, although the same quantity of yeast extract is completely lacking in toxicity for nonirradiated mice.

Figure 3 indicates results of tests on the effect of various concentrations of yeast extract on pea sprouts irradiated at a dosage of 500 r.

From Fig. 3 it can be seen that the yeast extract lowers the percent of abnormal cell divisions by several times, and that there is also a noticeable decrease in growth depression. The good correlation between these effects and also the circumstance that the strongest protective effect is manifested on the cytological disturbances and the least on root growth suppression, is of interest. The optimum effect was found at a 1:1000 yeast extract dilution.

The results obtained are of considerable interest chiefly because as has been pointed out, little is known of the anti-irradiation remedies whose effects are exerted after radiation. In addition, the nonspecificity of the effect of the yeast extract is of interest – the yeast extracts showed effect on mice and on peas, while other tissue preparations showing an effect when used after irradiation have a high specificity [7].

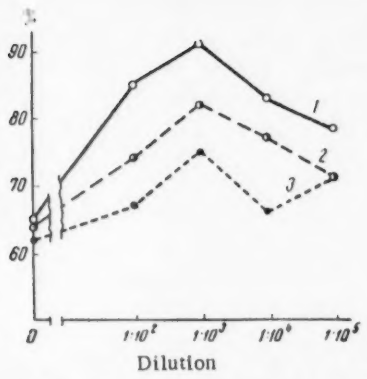

Fig. 3. Effect of various concentrations of yeast extract on percent of normal mitoses (1), entire aboveground portion (2), entire root (3) upon irradiation of pea sprouts at a 500 r dosage: values expressed in % of nonirradiated control.

As was noted above, a marked effect was not obtained in all the tests with yeast extract. Some extracts gave a lesser effect, and at times there was none shown. Accordingly, the most immediate task was to clarify the causes responsible for this phenomenon.

Effect of Harmful Agents on Yeast Extract Properties

Analysis of the tests conducted created the impression that the temperature at which the yeast was stored before preparation of the extracts had a considerable significance. Accordingly special tests were set up to elucidate the effect of temperature and other unfavorable conditions (drying and irradiation) on the yeast cells.

The results of several typical tests are given in Table 1. These tests showed that the temperature has a very considerable significance: the best results were

TABLE 1

Effect of Various Yeast Extracts on Mortality of Strain M Mice Irradiated with γ-Rays (dosage 500 r)

Type of yeast taken for preparation of extract	Addition	No. of animals	Mortality at 20 days		p
			no.	%	
Yeast stored at 8°	0.3 ml	12	10	83.3	0.5000
" " " 4°	0.3 ml	12	8	66.7	0.0330
" " " 8°	0.3 ml	12	12	100.0	0.5000
Control (without introd. of extract)	—	36	33	91.7	—
Dried bakers' yeast	4 ml	12	7	58.3	0.0775
Dried brewers' yeast	4 ml	12	6	50.0	0.0343
Control (without introd. of extract)	—	12	11	91.7	—
Nonirradiated yeast	4 ml	12	11	91.7	0.5000
Yeast irradiated at a 5000 r dosage	4 ml	12	6	50.0	0.0069
The same at a 25,000 r dosage	4 ml	12	8	66.7	0.0466
Control (without introd. of extract)	—	12	12	100.0	—

obtained upon storage of the yeast at 4°; if the yeast from which the extracts were obtained was held at a higher temperature or was kept in the frozen condition, effective extracts were not obtained.

Other harmful agents (drying, irradiation) also lead to some cellular changes, as a result of which anti-irradiation substances are formed. The similarity between the effects of temperature and irradiation are of particular interest. Thus, while cooling to low positive temperatures gave better results than freezing, in the same way irradiation at a dosage of 5000 r yielded better active extracts than irradiation with a 25,000 r dosage. It should be noted that in the tests conducted the irradiation proceeded for a day. If the same total dosage was at a given higher output for a shorter time, the extract obtained would not possess the same protective properties.

It is evident that the yeast cells which are subjected to unfavorable conditions are capable of forming or accumulating some substances which have anti-irradiation properties, which explains the different effects of various preparations.

Further tests described herein were devoted to clarification of the chemical nature of the source of effects of yeast extracts which decrease the harmful biological effects of irradiation.

Chemical Nature of Active Source

The characteristics of yeast extracts stimulating cell division have already been studied. It is known that among them nucleic acid derivatives [8], amino acids [9], and B group vitamins [10] play a role. Accordingly, in experiments to clarify the chemical nature of the active source of yeast extracts, these substances were studied first.

TABLE 2

Protein Determination in Yeast Extracts

Preparation	Color reaction			Precipitation reaction		
	ninhydrin	biuret	xanthoproteic	sulfoalicyclic acid	nitric acid	chloroform
Extract 1	++++	+	?	—	—	+
" 2	++++	+	?	±	—	+
" 3	++++	++	?	+	?	++
" 4	++++	+	?	±	—	+
" 5	++++	+	?	±	—	+
Casein hydrolyzate	++++	±	—	—	—	±
Albumin	+	++++	++++	++++	++++	++++

Note. Results evaluated visually. It is difficult to judge the results of the xanthoproteic reaction because of the yellow color of the extracts themselves.

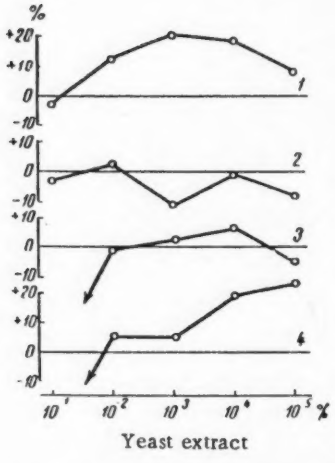

Fig. 4. Effect of various concentrations of yeast extract (1), amino acid mixture (2), mixture of B vitamins (3), and ribonucleic acids (4) on growth of peas, irradiated at a 600 r dosage: data expressed in % deviation from the control, irradiated at the same dosage.

There were used: an enzymatic casein hydrolyzate; a mixture of B group vitamins in approximately the proportions in which they are contained in yeast; yeast nucleic acids and yeast extracts. The degree of suppression of growth of peas irradiated in the sprout stage at a 600 r dosage served as the criterion of effectiveness. All the substances were used in a broad range of concentrations.

From Fig. 4 it can be seen that neither the amino acids nor the vitamins at any of the concentrations used reduced the substantial effects of irradiation. As for RNA, it exerted the same quantitative effect as the yeast extract in that the optimum action was at a lower concentration.

Special tests were set up to exclude the possible role of proteins in lowering the irradiation effect. First the protein, peptide and amino acid content of several samples of yeast extract was determined.

The results of these analyses, given in Table 2, show that the proteins in the yeast extracts used are almost completely hydrolyzed to amino acids. As to the ineffectiveness of amino acids, this had already been established above.

Additional tests were made in which the effects of ordinary yeast extracts, and the same extracts purified of traces of protein, according to Sevag [11] were compared on irradiated mice. As indicated in Table 3, both extracts were shown to have the same effect. Thus, it can be fairly definitely concluded that the yeast extract proteins do not exert any anti-irradiation effect, and the only substances determining activity of yeast extracts are the ribonucleic acids.

TABLE 3

Effect of Yeast Extracts Purified of Protein Traces on Mortality of Strain K Mice Irradiated at a 600 r Dosage

Test conditions	Addition in ml	No. of mice	Mortality at 20 days		P
			no.	in %	
Original extract	0.3	10	3	30.0	0.0894
Control (without extract)	—	10	7	70.0	
Purified extract	0.3	10	3	30.0	0.1849
Control (without extract)	—	10	6	60.0	

Role of Ribonucleic Acids

As indicated above, the first tests with RNA on rats gave negative results, which was at variance with results obtained in tests up to that time. Therefore, tests were set up on mice in which the effect of 2 RNA preparations were compared.

TABLE 4

Effect of Various Ribonucleic Acid Preparations on Mortality of Strain M Mice, Irradiated at 500 r

Substance introduced	Mg added	No. of mice	Mortality after 20 days		P
			no.	%	
Original extract	4	12	7	58.3	0.0775
Separated RNA	1	12	6	50.0	0.0343
Control	—	12	11	94.7	—

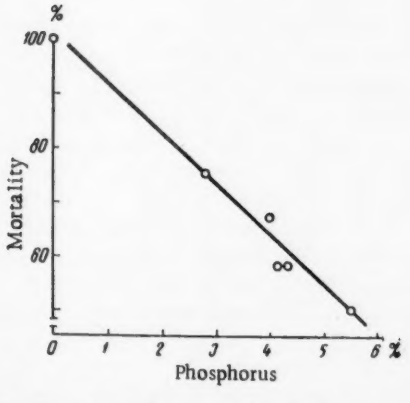

Fig. 5. Correlation between phosphorus content (in %) of yeast extracts and their protective effect: tests on strain M mice irradiated at 500 r.

TABLE 5

Effect of RNA Separated from Yeast Extract on Mortality of Strain M Mice, Irradiated at 500 r

RNA preparation	Mg added	No. of mice	Mortality after 20 days		P
			no.	%	
1	0.1—1	12	11	91.1	
Control (w/o RNA)	—	12	10	83.3	0,5000
2	0.1—1	12	3	25.0	
Control (w/o RNA)	—	12	10	83.3	0.0061

It was shown (Table 4) that a preparation which gave negative effects on rats had the same results on mice, and that a preparation which decreased the radiation disease in peas significantly lowered mortality of irradiated mice. Thus, the difference is explained by some difference in RNA preparations.

To exclude the possible role of other protein extract components, tests were conducted in which the effectiveness of yeast extracts and ribonucleic acid separated from those extracts were compared. The separations were made by precipitation with ethyl alcohol in acid medium with subsequent purification by organic solvents.

The results of these tests, given in Table 5, showed that RNA separated from yeast extracts has the same effect as the whole extract.

If the anti-irradiation properties of yeast extract are related to the presence of nucleic acid, their effec-

tiveness should correlate with their phosphorus content since, with water extraction, the main mass of phosphorus in the extracts is nucleic acid phosphorus. Accordingly, tests were set up in which 5 samples of yeast extract with various phosphorus concentrations from 2.8 to 5.5% were compared as to their effect on mortality of irradiated mice.

The results shown in Fig. 5 showed that there was a close correlation between phosphorus content and anti-irradiation effectiveness of the extracts.

Thus it is evident that the anti-irradiation effect of yeast extracts is related to their RNA content. Confirming this, none of the yeast extract components except nucleic acids diminished the biological effects brought about by irradiation. RNA shows a similar effect to yeast extract under the same conditions, and both the RNA separated from the extract and obtained in preparations as usually prepared from yeast were effective. In this respect it should be noted that not all RNA preparations have the same properties. The reasons for such differences are of particular interest and merit special attention.

DISCUSSION OF RESULTS

The tests described above indicate that yeast extracts and RNA decrease irradiation effects when they are used after irradiation.

Until now only the therapeutic effects of antibiotics [12], spleen preparations [7], and bone marrow [13] could be referred to. Some other reports as to the therapeutic effect of one or another substances were not confirmed by other workers (see for example [14] and [15]). The antibiotic effect is quite trivial, inasmuch as this concerns decreasing the role of infection, which is important in an organism weakened by radiation sickness. As for spleen and bone marrow preparations, attention must be given to the fact that these organs are particularly rich in nucleic acids.

In this connection, there occur analogies between the effects of the substances indicated and the results of our tests with yeast extract and nucleic acids. However, in making these comparisons, a number of contradictions are observed. The spleen preparations are effective only in using strictly homoplastic material [7]; weak denaturation of these materials leads to a loss of anti-irradiation properties [16]. In our tests, on the other hand, heteroplastic materials were shown to be effective and an injurious effect strengthened the anti-irradiation properties. Further, although in tests with spleen preparations it was established that the source of activity was the nucleoproteins of the cell nucleus, isolated nucleoproteins were ineffective [17]. In our tests RNA gave the same effect as yeast extracts.

In our opinion, all the available experimental data can be explained on the basis of a single hypothesis.

Analysis of the composition of nucleic acids from various sources indicates their species specificity [18], which is in accordance with the current opinion as to the role of nucleic acid in the storage of hereditary information [19] and the determination of a series of amino acids upon synthesis of specific proteins [20]. Accordingly, if introduction of nucleic acids or nucleoproteins into the organism compensates for an insufficiency of these substances originating as a result of irradiation, then strictly homoplastic native materials should result in the best effects. This is actually observed. In the most successful tests with the yeast extract, the $LD_{50/30}$ can be reduced only by 20%, while implantation of homoplastic materials lowers it to about half [7, 13]. Weak denaturation of homoplastic materials or native heteroplastic materials need not be effective, since in these cases alien nucleic acids are introduced which the organism cannot use directly. However, strong denaturation in which the species specificity of nucleic acids is lost, should yield effective material, although less effective than the native specific nucleic acid. Species nonspecific products of degradation of nucleic acids may serve as a good "structural material" for the synthesis of specific nucleic acids. The question as to whether degradation to the mono- or only to oligo nucleotide is necessary remains unclear. The fact that mono nucleotides have a very weak effect in radiation disease [21, 22] is the basis of the supposition that larger "blocks" may be used in biosynthesis of nucleic acids. This possibility, the experimental proof of which will be the subject of a special study, is of special significance in the problem of autoreduction of biologically important macromolecules, the more so that there is already some data both theoretical [23] and experimental [24] indicating that autoreduction may proceed not via monomers but by larger molecular aggregates.

SUMMARY

Yeast extracts have the property of decreasing the harmful effect of radiation on the living organism. They differ advantageously from most other anti-irradiation remedies: in showing effectiveness upon use after irradiation and not possessing the species specificity characteristic of other tissue preparations which are effective after irradiation, such as spleen and bone marrow.

In tests with mice, yeast extracts increase LD_{50} by about 100 r. In tests on pea sprouts they lower the percent of pathological mitoses several times and decrease the depression of plant growth brought about by irradiation.

Not all yeast extracts have the same effectiveness. The conditions of storage of the yeast before preparation of their extracts are factors of considerable importance determining their effectiveness. The most effective extracts are obtained following action of various agents injurious to the yeast (cold, drying, irradiation) in which the optimal conditions are those which depress but do not halt living processes (thus cooling to low positive temperatures gives better effects than freezing; irradiation with 5000 r a better effect than 25,000 r).

The effective source in yeast extracts is RNA or its derivatives. Water soluble vitamins, amino acids and proteins do not play a significant role. The effectiveness of yeast extracts is correlated with their phosphorus content. RNA, both separated from yeast extracts and obtained preparatively, has the same effect as yeast extract; however, some RNA preparations are ineffective.

The hypothesis is formulated that radiation damage to nucleoprotein metabolism can be decreased by the introduction either of specific nucleic acids or of depolymerized nucleic acids which have lost their species specificity, while denatured or foreign polymerized nucleic acids have no effect. In such cases the results described are in accordance with data as to the anti-irradiation effect of spleen and bone marrow preparations.

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CHANGES IN THE COMPOSITION OF NUCLEIC ACIDS IN THE FORMATION OF SACCHAROLYTIC INERT FORMS OF INTESTINAL BACTERIA

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At the present time a large body of experimental facts shows the close connection of the nucleic acids of cells with heredity and species specificity [1]. Bacteria demonstrate this particularly well.

In this group of organisms it has been shown that the nucleotide composition of DNA does not depend on growth or physiological condition of the cells, but is determined only by the species individually [2-5]. It was shown that the composition of DNA in divergent species could differ greatly, and on the contrary, biologically closely related forms of bacteria had, as a rule, similar composition in their DNA [4, 5]. It was also shown that the composition of bacterial DNA could be greatly changed by sharp experimental variations connected with fundamental reorganization of all enzyme and antigen complexes of the bacterial cell [6]. Such great species differences were not noted in the nucleotide composition of RNA [5, 7]. In the deep hereditary variations which accompany changes in DNA composition, such changes were not found in the composition of RNA [6].

Such data led two of us to assume that if DNA had such specific functions, RNA, or in the cases considered, the cellular materials which contained it, had a more general and less specific function [5]. It should not be forgotten, however, that a small part of the cellular RNA has a nucleotide composition closely related to that of DNA [5] and so, along with DNA must have a direct relation to the specificity of the organism.

The purpose of the present work was to continue the study of the relation of nucleotide composition of DNA and RNA to deep changes in the heredity of the cell.

We confined our experiments to bacteria, since these organisms most easily undergo deep hereditary changes.

There is also a large microbiological literature on experiments with bacteria which have genetically stable forms obtained experimentally by various treatments and which differ sharply from the starting bacteria in their morphological, cultural, serological, and biochemical properties. These include different L-forms and G-forms. Naturally, the question arises as to whether the nucleic acids have changed in these variants. In turn, analysis of specificity in composition of the nucleic acids should help to decide whether visible changes in the bacterial cell are connected with genetic changes or are only changes in phenotype.

MATERIALS

We had at our disposal the so-called "saccharolytic inert" form obtained from intestinal bacteria by various types of unfavorable treatment [8, 9]. These "saccharolytic inert" forms could be divided into two groups in accord with their properties. One group was conditionally designated the "neutral form" since these organisms gave a neutral reaction in a Hiss peptone medium with carbohydrates. They are evidently identical with the form described in the foreign literature as the G-form. The other group was designated the "alkali-producing form," since this variant made the Hiss medium alkaline as it grew.

We used the following bacterial cultures in our investigation.

1. The intestinal bacteria, Bact. coli commune (E. coli), Strain No. 1. Taken from the contents of rabbit intestine.

2. "Neutral" regenerating, filterable form Cl-F4. Obtained from Bact. coli I by long maintainance in distilled water, followed by filtration through a bacterial filter and inoculating the filtrate in Martin broth.

3. "Alkali-producing" form C-1-A. Obtained from E. coli I by long maintainance in distilled water, followed by seeding in a meat-peptone broth (MPB).

4. Abdominal type bacteria, Salmonella typhosa, Strain Ty-2. Taken from the collection of live cultures of the Gamaleia Institute of Epidemiology and Microbiology of the Academy of Medical Sciences of the USSR.

5. "Neutral" form Ty-10-N. Obtained from S. typhosa by prolonged culturing (more than a month) in a meat broth with pH 8.4 without reseeding, but with periodic repeated shaking on a shaking machine, then followed by seeding on MPB.

6. "Alkali-producing" form Ty-10-A. Obtained from "neutral" form Ty-10-N in one of its seedings after transfer to a medium with a protein substrate as the sole source of nitrogen.

7. Dysentery bacteria, Shigella dysenteriae, Strain No. 913. Taken from the collection of live cultures of the Gamaleia IEM of the Academy of Medical Sciences of the USSR.

8. "Alkali-producing" form 913-A-Moroz. Obtained by A. F. Moroz from Sh. dysenteriae No. 913 by the action of the antibiotic grisein.

9. "Alkali-producing" form 913-A-Mitereva. Obtained from Sh. dysenteriae No. 913 by V. G. Mitereva by prolonged culturing in MPB without reseeding and with periodic shaking on a shaking machine and subsequent seeding on Martin broth.

Before obtaining the saccharolytic inert forms from intestinal bacteria the starting cultures were reseeded ten times, each reseeding being tested by isolation of a colony and study of the properties of this subculture. Thus the homogeneity of the starting culture was tested, and the tenfold reseeding guaranteed a pure culture. After obtaining the desired form, the starting culture was tested weekly for the stability of its properties. The saccharolytic properties of the starting culture and the variants obtained from it were studied by seeding an agar culture daily on a Hiss peptone medium with bromothymol blue and with addition of 0.5% of one of five carbohydrates: lactose, glucose, mannose, maltose, or sucrose.

As an example, we give a short description of obtaining both "saccharolytic inert" forms from E. coli, the "neutral form" Cl-F4 and the "alkali-producing form" C-1-A [9].

Obtaining "neutral form" Cl-F4 from E. coli I. Weighed cells from a 24-hr culture of E. coli were maintained in distilled water for 4 months, after which the suspension was filtered through a sterilized asbestos filter. Part of the filtrate was seeded in Martin broth. As a control we used: 1) the same broth without the filtrate; 2) the same filtrate, without the broth. After 22 days incubation at 37° a slight cloudiness and fine precipitate were observed. Microscopic study showed the presence of distinct granules and also single fine gram-negative bacteria. In the control flasks there was no change in two months.

Seeding took place from the experimental and control flasks on MPB and on agar (MPA). The control flasks showed no growth on either medium, while the media seeded from the experimental flasks in a day at 37° showed a very weak growth. On the agar a very small colony was formed which in 48-72 hrs increased slightly in size. In the broth (MPB) a turbidity occurred and a precipitate settled. The culture did not form acid on the Hiss peptone medium with carbohydrates nor did these media become alkaline.

The regenerating, filtered form Cl-F4 thus obtained was passed frequently, in its daily transfers, through a highly nutritious medium and then again through a medium with ordinary MPA. The original very weak growth then became greater. The pleomorphic cells seen earlier under the microscope gradually disappeared, and after 20-30 transfers the cultures consisted of coarse, thick, weakly gram staining, nonmotile cocci. As a control the starting cultures were transferred to the same media, and through the entire series of transfers the strain of E. coli kept its own properties.

We should observe that when transference was continued, the "neutral" form showed tendencies toward two variations from its original properties: first toward formation of acid in the Hiss medium with carbohydrates (that is, reversion to the starting culture of E. coli), second toward formation of alkali on the peptone medium (that is, toward the "alkali-producing" form). These variations appear simultaneously with the appearance in the colony of another type of properties than the fine granular colony which is the "neutral" form.

The "neutral" variant was separated and submitted to a fivefold transfer. After each seeding the colony was chosen which gave a subculture with the original properties (neutral reaction on the Hiss peptone medium with carbohydrates). The selected subcultures served for the next seeding. In the first seeding the culture was nonhomogeneous: besides the "neutral" form there were colonies which gave acid-forming subcultures. The second subculture, however, gave only one variant subculture from ten experiments. In the last three transfers the 20 experimental colonies were homogeneous in every case. The rest of the cultures of the "neutral" form C1-F4 showed stability in properties on storage or on transfer.

Obtaining the "alkali-producing" form C-1-A from E. coli I. A 24-hr culture of E. coli I was seeded into a flask of distilled water. As controls, unseeded flasks of distilled water or MPB were used. Every week transfers were made from these flasks onto MPA and Endo agar. In 24 hrs no cells were found in the controls. In the experimental cultures after 16 weeks of culturing in distilled water and seeding onto Endo agar with good colonies typical of the initial intestinal bacteria, there began to appear fine, colorless colonies from which in some cases it was possible to obtain subcultures which made the Hiss peptone medium alkaline.

The alkali-producing variant C-1-A thus formed was submitted to five transfers. After the first transfer most of the colonies showed partial reversion to the starting culture (forming acid on the Hiss peptone medium with some carbohydrates). After selection of the alkaline colony and its transfer (second transfer), only a small part of the colony showed reversion. The three following transfers gave perfectly similar colonies of typical "alkali-producing" forms. After such selection the variant C-1-A remained unchanged on storage and on transfer.

This "alkali-producing" form like the "alkali-producing" forms from other intestinal bacteria appeared under the microscope as a motile, gram-negative, short rod with rounded ends. After a day's growth on MPA at 37° it formed a small, shining colony, round, with smooth edges, bluish in transmitted light. On the surface of the broth a delicate film and a ring on the vessel wall were formed. The cultures gave intensive growth at room temperature.

EXPERIMENTAL RESULTS

Biochemical Properties of the Starting and Experimentally Obtained Forms

Some biochemical properties and also the morphology and motility of the cultures which were studied are given in Table 1.

Even from Table 1 it is clear that the "alkali-producing" forms of different intestinal bacteria show similarities in their biological properties although they differ greatly from the starting cultures. Both "neutral" forms also comprise a single group of bacteria, and, although they are of different origin and are prepared by different methods (Ty-10-N was obtained without filtering the starting culture), they are similar to each other in properties. These two characteristics – great variation in properties compared with the starting culture and similarity of properties in the typical forms of different origins – are still more clearly seen by comparing the data on stability to antibiotics.

The data in Table 2 show the exceptional stability of the alkali-producing forms toward penicillin and also to streptomycin, and, on the contrary, their extreme sensitivity to aureomycin. The "neutral" forms are characterized by increased sensitivity to all the antibiotics studied. These results show first that the alkali-producing forms and the neutral forms are two entirely distinct groups of "saccharolytic inert form," and the second, that both the neutral and the alkali-producing forms are related to profound, but at the same time characteristic, changes in the total specific cellular metabolism.

TABLE 1

Some Properties of the Cultures Studied

Name of bacterial form	Morphology	Gram staining	motility	Gelatin liquefaction	Formation of			Behavior on Hiss medium with carbohydrate				
					indole	H_2S	NH_3	lactose	glucose	mannose	maltose	sucrose
Escherichia coli I (starting)	Rods	−	+	−	+	+	+	AG	AG	AG	AG	−
Neutral C1-F4	Cocci	±	−	−	−	−	−	−	−	−	−	−
Alkali-producing C-1-A	Rods	−	+	+	−	+	+	Al	Al	Al	Al	Al
Salmonella typhosa Ty-2 (starting)	Rods	−	+	−	+	+	+	−	A	A	A	−
Neutral Ty-10-N	Cocci	±	−	−	−	−	+	−	−	−	−	−
Alkali-producing Ty-10-A	Rods	−	+	+	−	+	+	Al	Al	Al	Al	Al
Shigella dysenteriae No. 913 (starting)	Rods	−	−	−	−	−	−	−	A	−	A	−
Alkali-producing 913-A-Moroz	Rods	−	+	+	−	+	+	Al	Al	Al	Al	Al
Alkali-producing 913-A-Mitereva	Rods	−	+	+	−	+	+	Al	Al	Al	Al	Al

Note. A − forms acid; AG − forms acid and gas; Al − forms alkali.

TABLE 2

Minimal Bacteriostatic Dose of Antibiotics in Units per ml of Medium

Antibiotic	Starting intestinal bacteria	Neutral forms	Alkali-producing forms
Penicillin	25	0.1-0.25	1000-2000
Streptomycin	1.5	0.1-1.0	50-250
Chloromycetin (syntomycin)	2-10	2-4	6-40
Aureomycin (biomycin)	12-25	0.5-1	0.5-1.5

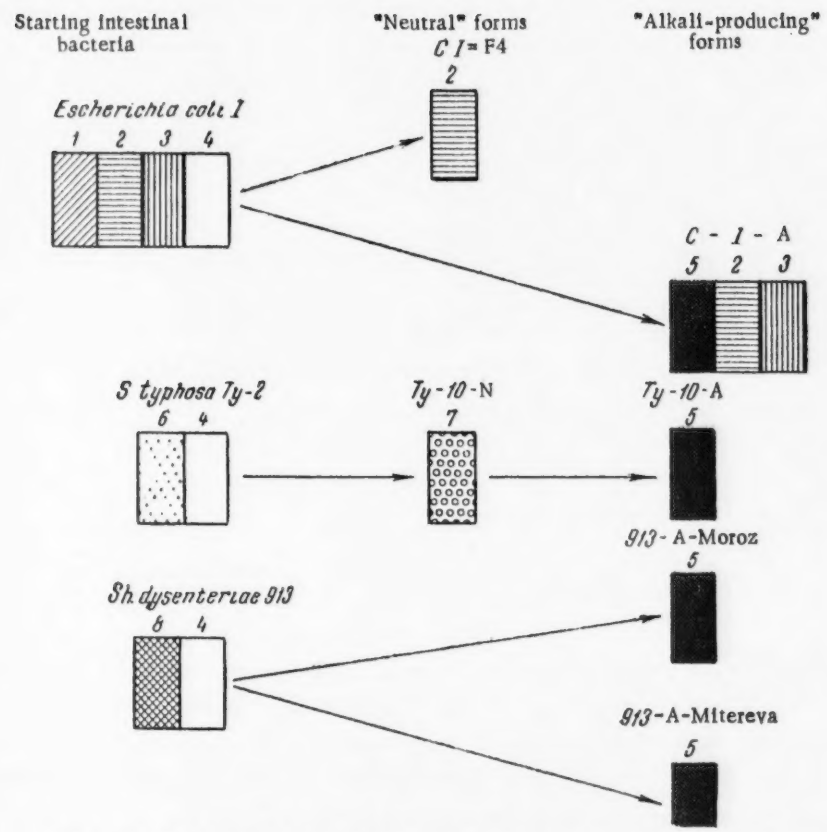
Antigen Structure of the Protein Fraction of the Cultures Studied

To discover the antigen composition, comparative immunological studies were made of the protein fractions obtained by fractionation of the bacterial mass of the cultures studied. Details of this study have been published elsewhere [10]. To summarize the results of these studies, the antigen composition of the cultures can be expressed schematically as shown in the figure.

Thus, the results of the immunological study show that in forming the saccharolytic inert form from intestinal bacterial there occurs as a rule a general reorganization of the whole antigen complex, that is, evidently, a profound change in the proteins of the specific cells. When the alkali-producing forms are obtained there is formation of a single type of antigen complex with identical serological specificity, independent of the starting culture.

DNA Composition of the Cultures Studied

The nucleotide composition of the DNA and RNA was studied by the methods which were described earlier [6], using more recently developed modifications [5, 7].



Scheme of the antigen structure of starting and experimentally obtained forms: 1, 2, 3) antigens characteristic of E. coli; 4) total group of antigens of intestinal bacteria (E. coli, S. typhosa, Sh. dysenteriae, and S. typhimurium); 5) antigens specific for the alkali-producing form (same antigen group); 6) antigens specific for S. typhosa; 7) antigen specific for form Ty-10-N; 8) antigens specific for Sh. dysenteriae.

The data on the composition of DNA presented in Table 3 clearly show that there are three groups of DNA composition in the cultures studied: 1) the starting forms of intestinal bacteria with a weak GC type $\left(\frac{G+C}{A+T} \simeq 1.1\right)$; 2) the alkali-producing forms which, independent of source, and method of preparation, have a strong GC type $\left(\frac{G+C}{A+T} \simeq 2\right)$; 3) neutral forms which have the AT type of DNA $\left(\frac{G+C}{A+T} \simeq 0.7\text{-}0.8\right)$. It is important to mention, however, that in spite of the similarity in composition of DNA within the limits of each group, full identity of DNA composition does not occur among the forms of one type. This shows the possibility of some individual specificity in the case of some of the cultures studied.

At any rate, the analysis of the composition of DNA makes it very clear that, first, the neutral and alkali-producing forms make up two entirely different groups of the "saccharolytic inert" form; second, the transformation of the intestinal bacteria into the neutral or alkali-producing forms is accompanied by a very great shift in the specificity of cellular DNA; third, this shift is typical of each group in the limits of the "saccharolytic inert" form.

TABLE 3

Ratio of Bases in the DNA from Starting Intestinal Bacteria and the Forms Obtained Experimentally

Name of bacterial form	Content of bases in mole%				Pur./Pyr.	G + T / A + C	G + C / A + T
	G	A	C	T			
Escherichia coli I (starting)	26.0	23.9	26.2	23.9	1.00	1.00	1.09
Neutral Cl-F4	21.0	29.2	20.6	29.2	1.01	1.01	0.71
Alkali-producing C-1-A	33.3	16.7	33.8	16.2	1.00	0.98	2.04
Salmonella typhosa Ty-2 (starting)	26.7	23.5	26.4	23.4	1.01	1.00	1.13
Neutral Ty-10-N	21.9	28.2	21.7	28.2	1.00	1.00	0.77
Alkali-producing Ty-10-A	32.2	17.3	32.4	18.1	0.98	1.01	1.83
Shigella dysenteriae 913 (starting)	26.7	23.5	26.7	23.1	1.01	0.99	1.15
Alkali-producing 913-A-Moroz	32.8	17.0	33.2	17.0	0.99	0.99	1.94
Alkali-producing 913-A-Mitereva	32.8	16.7	33.9	16.6	0.98	0.98	2.00

Note. G – guanine, A – adenine, C – cytosine, T – thymine; Pur. – purine bases, Pyr. – pyrimidine bases.

It is important to relate these conclusions to the earlier data on change in DNA composition in experimental variability of intestinal bacteria under the action of dead breslau paratyphus bacteria [6].

In that work a study was made of the "alkali-producing II-IV-4," formed from intestinal bacteria by raising them on a medium which contained as its sole source of nitrogen dead breslau paratyphus bacteria, which had the same DNA composition as the alkali-producing forms reported here $\left(\frac{G+C}{A+T} \simeq 2\right)$.

This "alkali-producing II-IV-4" was completely similar to the alkali-producing forms described here in all its other properties: fermenting and biochemical activity, morphology, relation to antibiotics and chiefly in its antigens [11, 12]. Hence, it is evident that this formation from intestinal bacteria is not due to any specific "directing" action of the dead breslau paratyphus organisms, but simply is the result of transfer of the intestinal bacteria into very unfavorable conditions – conditions of forced assimilation of a protein substrate.

Thus our data permit us to treat the first stage of the so-called "directed variability" of intestinal bacteria as their transformation into an alkali-producing form by the action of unfavorable conditions. On further contact of the alkali-producing forms with the dead breslau paratyphus bacteria there evidently occurred, in the experiment, reversion to the properties of the intestinal bacteria. At the same time the composition of the DNA reverted to the original [6]. Here then, evidently, the dead breslau paratyphus bacteria could play the specific role of a "directing" substrate, since the form obtained by reversion, paratyphoid 12-IV-4, resembled in antigen condition and some other properties the breslau paratyphus bacteria [11, 12]. This more precise treatment of the previous experiments does not in any way change them, but only confirms the conclusions which were drawn from the biochemical study [6, 11] and particularly the conclusion that DNA changes with change in hereditary specificity of bacteria [6].

Comparing our data on the composition of DNA of the cultures studied with the properties of these cultures, we find a series of regular correlations. Thus, all the starting intestinal bacteria were similar in biological properties, in reaction to antibiotics, all had the same general group of antigens in the cells [10]. These biological similarities agreed well with the similarity in nucleotide composition of their DNA. The alkali-producing forms showed the same general relationship in biological properties, stability to antibiotics, and antigen structure to their similarity in DNA composition even though they were obtained by different methods and from different sources. The same was true for the neutral forms studied.

On the other hand, fundamental reorganization of antigen structure and other properties when the intestinal bacteria were transformed into alkali-producing forms was accompanied by a very considerable change in DNA composition. Thus a change in biological, serological or other properties of one type was unavoidably linked with a change in DNA composition. In all cases studied, of whatever starting intestinal bacteria, the gain of the ability to alkalize Hiss peptone medium and change the antigen complex was accompanied by a change in DNA composition toward the GC type $\left(\frac{G+C}{A+T} \simeq 2\right)$. Along with this clearly determined change, went change in all other biological properties. When a neutral form occurred, we also found a perfectly regular picture: when a definite set of biological and biochemical properties was acquired (neutral reaction on the Hiss medium with carbohydrates, increased sensitivity to antibiotics, characteristic cell morphology, etc.), the composition of DNA shifted to the AT type. It is true that here we did not observe such clear regularities in acquiring an antigen structure as in the alkali-producing form, but still a shift in antigen structure was very evident.

A good example of the correlation in DNA composition and specific cell metabolism of bacteria is given by comparison of Tables 2 and 3. It is clear that sensitivity to antibiotics (especially to penicillin) changes according to the ratio $\frac{G+C}{A+T}$ in the DNA of the cultures studied. In the case of penicillin, for example, the greater the value of (G + C/A + T), the higher the stability of the form to penicillin.

Thus, all the data lead to the conclusion of a close connection of DNA and specific cell metabolism, antigen specificity of cellular proteins, and specific hereditary cell behavior.

Nucleotide Composition of RNA in the Cultures Studied

The data on nucleotide composition of RNA are presented in Table 4 and show that change in composition of RNA with transformation of intestinal bacteria into the "saccharolytic inert" form is slight. Only in 2 or 3 cases

TABLE 4

Nucleotide Composition of RNA of Starting Bacteria and the Experimentally Produced Forms

Name of bacterial form	Nucleotide content in mole%				Pur./Pyr.	(G+C)/(A+U)	(G+U)/(A+C)
	G	A	C	U			
Escherichia coli I (starting)	30.7	26.0	24.1	19.2	1.31	1,21	1,00
Neutral C1-F4	28.9	27.1	23.2	20.8	1.27	1.09	0,99
Alkali-producing C-1-A	30.4	25.8	24.1	19.7	1.28	1,20	1.00
Salmonella typhosa Ty-2 (starting)	30.8	26.1	24.0	19.1	1.32	1.21	1.00
Neutral Ty-10-N	29.8	26.4	22.6	21.2	1.28	1.10	1.04
Alkali-producing Ty-10-A	30.7	25.7	24.3	19.3	1.29	1.22	1,00
Shigella dysenteriae 913 (starting)	30.4	25.9	24.4	19,3	1.29	1.21	0,99
Alkali-producing 913-A-Moroz	30.5	25.8	23.8	19.9	1.29	1.19	1,02
Alkali-producing 913-A-Mitereva	29.9	26.9	23.8	19.4	1.31	1,16	0,97

Note. G – guanylic acid; A – adenylic; C – cytidylic; U – uridylic; Pur. – purine nucleotides; Pyr. – pyrimidine nucleotides.

does the difference exceed the limits of experimental error. Thus it is seen that specificity in composition of RNA – the value of the ratio $\frac{G+C}{A+U}$ – is the same in all starting intestinal bacteria and in the alkali-producing forms obtained from them $(G + C/A + U = 1.20 \pm 0.02)$. Only form 913-A-Mitereva is a slight exception, since is shows a somewhat lower value, due to increase in adenylic acid content. Nonetheless, the composition of

RNA in the neutral form is quite different from that in the starting and alkali-producing forms.

Thus it is evident that with definite hereditary changes there can also be determinable changes in RNA composition, though not as considerable as in the case of DNA. It is characteristic that in the transformation to the "neutral" form the composition of RNA shifts toward a decreased value for the ratio $\frac{G+C}{A+U}$, that is, toward values like those of DNA when it changes from a weak GC type to the AT type. This is a fact – in line with experimental variability – which fits in with the earlier indication of a positive correlation in composition of RNA and composition of DNA [5]. Here, just as in the study of different forms of bacteria, the idea is suggested that only a small fraction of cellular RNA is correlated with DNA composition, so that only a small shift occurs in total nucleotide composition of RNA when there is a larger shift in DNA.

SUMMARY

A study was made of the nucleotide composition of DNA and RNA in hereditary stable saccharolytic inert forms obtained from intestinal bacteria by experimental variations of different types of unfavorable conditions (distilled water, culturing without transfer, antibiotics). The biological and biochemical properties, stability to antibiotics, and antigen composition of these forms were investigated at the same time.

It was shown that in properties and in composition of both nucleic acids the saccharolytic inert forms which were studied were clearly divided into two different groups of organisms, one of which was the so-called "neutral" form (evidently similar to that described in the literature as the G-form), and the other, the "alkali-producing" form.

It was shown that transformation of the intestinal bacteria into the "neutral" or the "alkali-producing" form was accompanied by a very great change in the nucleotide composition of DNA. The weak GC type of DNA in the starting bacteria $\left(\frac{G+C}{A+T} \simeq 1.1\right)$ changed in the "alkali-producing" form to a clear GC type $\left(\frac{G+C}{A+T} \simeq 2\right)$, and in the "neutral" form to the AT type $\left(\frac{G+C}{A+T} \simeq 0.7\text{-}0.8\right)$. These changes in DNA composition were closely correlated with the changes in all the hereditary properties – change in antigen structure, shift in enzyme complexes and altered sensitivity to antibiotics. On the other hand, forms closely similar in biochemical properties, stability to antibiotics, and antigen structure had similar DNA compositions.

It was shown that with such deep changes in biochemical and antigenic properties as occurred in the transformation of intestinal bacteria into saccharolytic inert forms, the nucleotide composition of RNA, in contrast to that of DNA, either did not change, or changed only slightly. Nevertheless, the change into the "neutral" form was accompanied by small but definite changes in RNA composition, with a decrease in the value of the ratio $\frac{G+C}{A+U}$.

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THE EFFECT OF SUBSTRATE AND ADENOSINE DIPHOSPHATE CONCENTRATION ON THE ACTIVITY OF MYOSIN ADENOSINETRIPHOSPHATASE

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The study of myosin ATPase kinetics started with the observation of ATPase activity in myosin [1]. The large body of experimental results published in the recent years indicate the continuing interest in this problem, but at the same time it is clear from the literature that the fundamental problems in the kinetics of myosin ATPase and the mechanism of its action are still far from final solution. This is also supported by the latest reviews on muscle biochemistry [2, 3].

Substrate inhibition of myosin ATPase was first established by Hasselbach [4], and the depression of its activity by ADP was shown by Kalckar [5] and confirmed by Green and Mommaerts [6]. However, these effects are still being questioned, while there is lack of agreement with respect to the evaluation of their qualitative aspect. Thus Blum [7] considers the inhibitory effect of ADP on myosin ATPase as quite insignificant. Green and Mommaerts [6],on the other hand, expressed doubts about the existence of the substrate inhibition effect. It has been correctly pointed out by Liubimova [8] that the fact that ADP can be prepared from ATP enzymatically in good yields, by dephosphorylation of the latter with myosin ATPase, contradicts the possibility of inhibition of myosin ATPase by ADP.

In this connection we have undertaken to study the substrate inhibition of myosin ATPase and the effect of ADP on its activity. The importance of enzyme concentration in the demonstration of these effects was also investigated.

METHODS

Myosin. Myosin was extracted from rabbit muscle with the solution described by Bailey (0.5 M KCl + 0.03 M $NaHCO_3$) in the cold for 10 min; the ratio of solution volume to muscle weight was 3:1. The myosin was precipitated three times with ten volumes of water at pH 6.8. Actin was removed according to Szent Gyorgyi by precipitation from the dilute solution at pH 8.3. The myosin solution, containing 2.0-3.0 mg N/ml, Q_p = 2000-3000, was kept in the refrigerator at 4-5°. The activity of the myosin fell gradually with storage, but no difference in experimental results, as compared with a freshly prepared solution, could be observed in the aged preparation.

The well-known thermolability of myosin in dilute solutions must always be kept in mind. Myosin removed from the refrigerator was always diluted with cold 0.5 M KCl immediately prior to use. When kept at room temperature in dilute solutions, myosin was rapidly inactivated, perceptibly so within one hour. This property of myosin has been reinvestigated recently by Quellet, Laidler and Morales [9], who confirmed the high thermolability.

The other circumstance which must be kept in mind is the homogeneity of the protein in solution. Myosin flocs form very easily on shaking, and we have therefore employed a special stirrer, consisting of a perforated plexiglas disc fixed on a glass rod. The stirrer, whose diameter was adjusted to that of the test tube, allowed rapid stirring of the myosin solution without frothing. Before the stirrer was devised, myosin solutions had to be centrifuged in the cold.

Protein concentration in most cases was 0.093 mg/ml (0.015 mg N/ml).

ATP. The preparation employed in the experiments was free ATP supplied by the Ivanov Meat Combine, prepared according to Liubimova [10]. Originally this was further purified in this laboratory by alcohol precipitation of the ATP in the cold, in order to remove inorganic phosphate contamination. Such a preparation contained other impurities derived from muscle (adenosine pentaphosphate, guanylic nucleotides), as well as ATP decomposition products, formed during preparation and storage (ADP, AMP, pyrophosphate and inorganic phosphate). The myosin-lability of crude ATP preparations varied, on a weight basis, between 65 and 80%, depending on the batch and duration of storage. The chemical analysis of components after separation by paper chromatography showed that the preparations consisted approximately of 70% ATP and 16-19% ADP [7]. ATP was therefore purified by chromatography on ion-exchange columns as described by Cohn and Carter [11], and only the purified preparations were used in subsequent experiments. Although preparatory separation of nucleotides is employed by many workers, as well as by firms which market biochemical preparations, the present authors are not aware of any detailed description of the procedure in the literature. It was therefore thought worthwhile to describe the procedure employed in the present work.

In the purification of ATP the resin employed was the powerful anion-exchanger De-acidite FF,* which is the British analog of the US product Dowex-2. 500 mg of commercial ATP was dissolved in 50-100 ml of N NH_4OH and passed through the column of the ion-exchanger (column dimensions, 10 × 50 mm, or 20 × 30 mm, Cl-form, mesh size 100, rate of liquid flow 1 to 3 ml/min). The column was washed to neutral eluate with water and the following eluants were passed through, 10 ml fractions being collected with a fraction collector:

1) 0.001 M NH_4Cl – 150-200 ml;

2) 0.003 M HCl; this solution eluted AMP as soon as the pH of the eluant, slowly shifting in the acid direction, reached about 3.0. The first 300-400 ml of this fraction need not be collected in small aliquots and the collector need only be positioned when the pH becomes slightly acid to universal indicator. The total volume was 600-700 ml;

3) 0.01 M HCl + .02M NaCl; this solution eluted ADP; total volume was 200-300 ml;

4) 0.01 M HCl + 0.2 M NaCl; this solution removed ATP; about 200 ml was used.

The optical density of all solutions was measured at 260 mμ in cells with a 10 mm light path in a SF-4 spectrophotometer; the molecular extinction at pH 2 was taken as 14,200. ATP was precipitated from the pooled fractions in solution (4), the highly concentrated fractions only (usually with E_{260} not below 30.0) being pooled. The pooled fractions were precipitated with ten volumes of cold ethanol according to Liubimova [9]. After vigorous stirring the highly turbid solution was left overnight, after which the precipitate was filtered off with suction, washed with cold ethanol, then with absolute alcohol, and dried in vacuo over $CaCl_2$. The yield varied between 250-350 mg.

The following remarks are of importance:

1. While Riner mesh of the ion exchanger assists the separation, it also slows down considerably the flow of liquid. We found 100 mesh quite satisfactory for preparative purposes.

2. N ammonia ensures more complete dissociation of the ATP and should therefore aid its adsorption by the ion-exchanger [11, 12]. Without any supporting quantitative evidence, the authors have found generally neutral and mildly alkaline solutions quite satisfactory for complete ATP adsorption.

3. Elution rates, varying from 0.5 to 3 ml/min were tested; the authors gained the impression that this factor was not critically significant in the preparative separation procedure. A fast rate of eluant flow has been preferred in recent experiments.

4. Column dimensions (internal) varied greatly – 10 × 50, 20 × 30, and 25 × 40 mm. The middle combination, 20 × 30 mm, which allowed fast flow, was found most convenient. The limiting capacity for such a column approaches, apparently, very closely 500 mg ATP.

5. The uniformity of packing and a perfectly horizontal surface, both on the top and bottom of the column, also appear to be of great importance. Departure from this rule leads to bad separation and distorted fronts. This results in overlapping of peaks and bad resolution of the components. This becomes less important under conditions

* Manufactured by the firm Permutit, London.

of good column resolving power, but affects the purity of preparations when the column is loaded to near-capacity and the various zones are close together.

Uniform packing may be obtained if the resin is allowed to settle in the column from a comparatively large volume of water. This is achieved by adding on, with the aid of a piece of rubber tubing, a length of glass tubing to the column to increase its capacity 3-4 times. The stirred suspension of the resin in a large volume of water is then poured as rapidly as possible into the elongated column assembly. The column should be held in the stand strictly vertically and the resin allowed to settle slowly.

A horizontal lower surface was secured by the position of the fixed bottom plate. Although the upper surface of the resin is always horizontal if packing is carried out correctly and the column held vertically, it should be protected against the disturbing action of the falling eluant drops. This was usually achieved by leaving a considerable column of water above the upper surface of the resin and lightly packing part of the remaining free space with fine glass wool.

6. The correct sequence of the eluting solutions must be adhered to for satisfactory resolution and the elution must be started with NH_4Cl. When the separation procedure is properly established, fraction collection may be started after the first 100 ml of the 0.01 M HCl-0.02 M NaCl solution has passed through, in order to make sure of the complete removal of the ADP by optical density measurements on the fractions from the second 100-ml portion of this eluant. The solution of 0.01 HCl-0.2 M NaCl is then collected in the usual manner for the isolation of ATP.

7. When the procedure is well organized the entire chromatographic procedure for ATP purification may be completed in one day.

8. The column is regenerated with 2N HCl and washed with water to neutral eluate.

9. The calculation of yields in the chromatographic procedure was carried out as follows. Instead of gravimetric data on the ATP present, the value used in the calculation was expressed as ΣE_{260} and may be referred to as the total extinction. This is obtained by multiplying the optical density at 260 mμ of any solution by its volume. It is easily seen that this value, which may at first appear to be meaningless, is indeed real. If the progress of separation be imagined as a graph, where the abscissa shows volume of eluant, while the ordinate shows the optical density, then ΣE_{260} corresponds to the area of the column which represents the given fraction on the graph. The sum of the ΣE_{260} values of all the columns equals the ΣE_{260} value of the original solution. In other words, the case represents an integration, carried out by simple arithmetic, and leading to results very similar to those, obtained by densitometry of chromatograms with subsequent integration of the curves obtained. It is possible, with the use of simple calculations, to convert ΣE_{260} into gravimetric values of ATP, ADP, adenine, etc. Apart from this, it is possible to express ΣE_{260} in the form of the optical density of a solution 1 ml in volume, but the result here is the same as above.

An example of a separation of commercial ATP is shown in Fig. 1.

ADP. It is possible to obtain 80-100 mg ADP, as a by-product in the purification of ATP, if the AMP, which is eluted with 0.003 M HCl, is carefully removed. It is, however, more convenient to obtain ADP specially.

The method used in the present work was that developed by Liubimova and Pevzner [13]. The method itself produced very satisfactory results but the final product depends to a large degree on the purity of the starting material. Faultless preparations of ADP may be obtained with the use of column chromatography. The procedure employed was as follows.

500 mg of ATP was dissolved in borate buffer, pH 9.0. The pH was readjusted to 9.0 on the glass electrode with the necessary amount of 2N KOH. The ATP solution was mixed with 30 ml of myosin solution (0.5 mg N/ml) and 10 ml of 0.05 M $CaCl_2$ and incubated at 37° for 30 min. The protein was precipitated with 25 ml of 20% trichloroacetic acid and the suspension filtered under gravity or centrifuged. The filtrate was adjusted to pH 6.8 by the addition of about 14 ml of 2N KOH. Twenty ml of 25% barium acetate and 150 ml (an equal volume) of ethyl alcohol was then added to the filtrate. The pH was again checked with bromothymol blue and, if necessary, adjusted by the addition of 2N KOH. It is advisable to leave the suspension overnight in the refrigerator, but it may be centrifuged after cooling on ice for 30-40 min. The supernatant was decanted and the suspension centrifuged. The precipitate was resuspended in 30 ml of water and about 3 ml of 2N HCl added to facilitate solution. A small amount of insoluble residue was discarded. The addition of about 4.8 ml of 2N KOH to the solution brought the

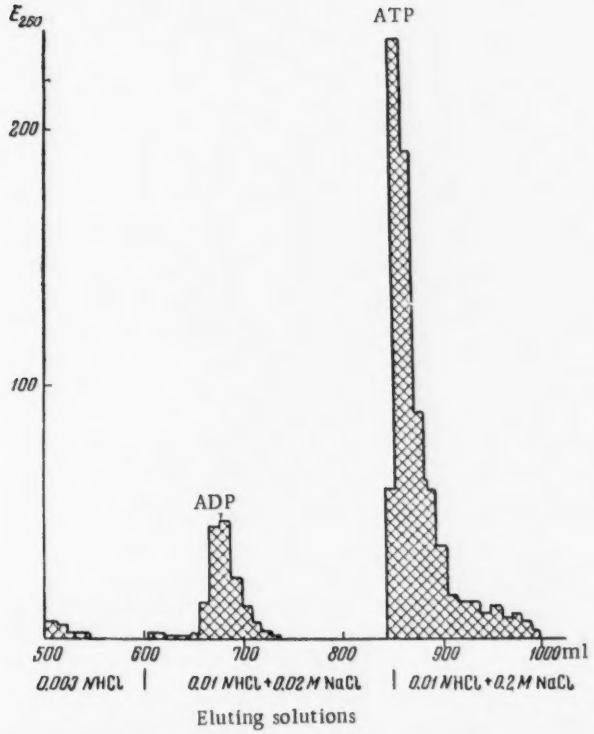

Fig. 1. Column chromatography of a commercial ATP preparation. Column: De-acidite FF, Cl-form, 100/mesh, dimensions 10 × 60 mm, flow rate 1 ml/min. Adsorbed 500 mg ATP (ΣE_{260} = 11,000). Eluted, ADP – ΣE_{260} = 1530 (14%); ATP – ΣE_{260} = 7470 (68%). Yield, about 300 mg.

pH to 6.8 (the total volume was then about 50 ml). Precipitation was repeated at pH 6.8, but only 0.5 ml of the barium acetate was added, followed by 50 ml of alcohol, and the pH again corrected. The suspension was left overnight in the refrigerator, or cooled for 30-40 min on ice, decanted and centrifuged. The precipitate was washed twice with 10 ml of 50% ethanol. All above operations were carried out in order to free the preparation from various anions, particularly Cl^-, which would be especially unwelcome in a separation on a column in the chloride form.

This procedure was followed by the removal of Ba^{++} and other cations. The cation-exchanger, KU-2, may be used successfully in this operation; this is a highly acid, monofunctional ion-exchanger, containing SO_3H groups. The washed ADP precipitate was suspended in about 25 ml of water, taking care to obtain a very fine suspension without coarse particles. About 7 ml of the swelled cation-exchanger (mesh 100, H-form) was added to the suspension. The precipitate was dissolved, giving rise to a cloudy solution. The solution was left for 20-30 min in the refrigerator. There are two possible avenues of further treatment. The resin may be centrifuged off and washed three times in the centrifuge with 10 ml of distilled water. Alternatively, the resin may be transferred to a column and washed on the column with 30-50 ml of water. In either case the pooled solution and washings were treated with concentrated ammonia – 1.4 ml to the total volume for neutralization of the ADP and a further 6.6 ml to each 100 ml solution (calculated for 29% ammonia). Since the solution obtained is slightly opalescent, it is advisable to leave it overnight in the refrigerator and then filter. Even this was not always satisfactory. The further steps were as described for ATP, but extreme care was taken in the elution with 0.003 N HCl solution in order to remove all traces of AMP, and the fractions obtained with the eluate 0.01 N HCl-0.02 M NaCl were collected. ADP is more soluble in the presence of ethanol than is ATP, and precipitates at a much slower rate. The yield was 200-300 mg. The chromatographic separation of ADP is shown in Fig. 2. It should be noted that the

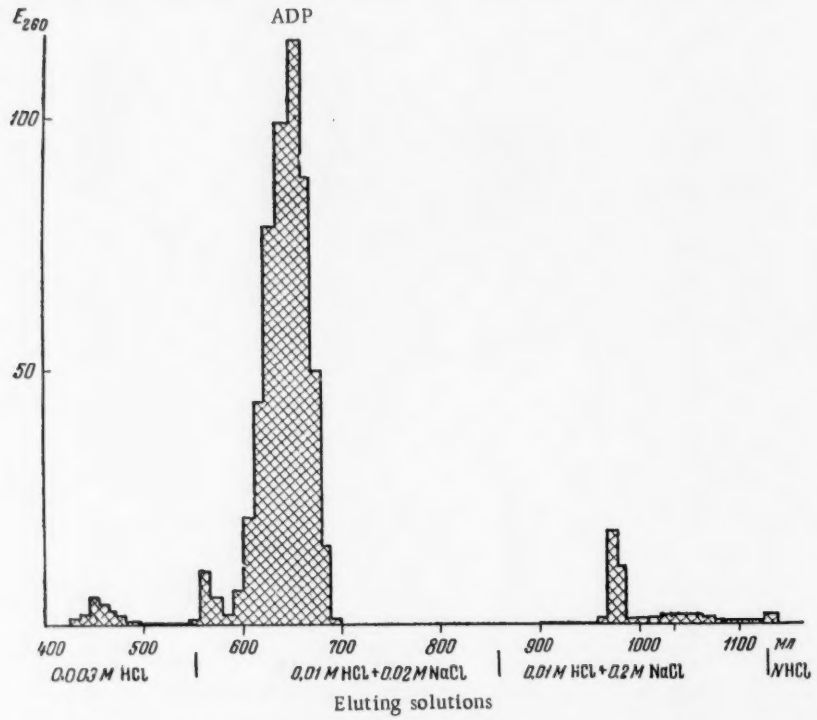

Fig. 2. ADP purification on ion-exchange column. Column: De-acidite FF, Cl-form, 100 mesh, dimensions 10 x 50 mm. Treated with myosin-500 mg crude ATP (ΣE_{260} = = 11,050). Recovered from KU-2 – ΣE_{260} = 8025 (73%). Eluted from column total of ΣE_{260} = 7600 (69%), including ADP (ΣE_{260} = 6760) – 61% of starting material.

enzymatic hydrolysis of ATP by myosin was nearly complete, since the total extinction at 260 mμ of the ATP fraction together with the guanosine nucleotides did not exceed 5% of the total in the case shown in Fig. 2.

Purity check on the ATP and ADP preparations. This was carried out using a paper chromatographic method developed in this laboratory, with the solvent n-propanol–ammonia (25%)–water (60:30:10). Inorganic phosphorus content did not generally exceed 2-4 μg P per mg preparation. Trace amounts of guanosine nucleotides were found in some of the ATP preparations. Adenosine pentaphosphate contamination, mentioned by Liebecq [12], could not be detected in any of the preparations. Small amounts of AMP were sometimes detected in some of the ADP preparations. In the authors' opinion this was due to the distortion of the front of the corresponding component during column separation. An example of a chromatogram of purified ATP and ADP samples is shown in Fig. 3.

The determination of the concentration of the ATP and ADP solutions. The concentration of ADP solutions was initially determined on the basis of the known amount used, corrected, in the case of impure preparations, for their myosin-lability; later the value of E_{260} was used exclusively, the molecular extinction coefficient being taken as 14,200 [12]. In all cases where crude ATP preparations were used, specific mention is made in the text, the actual concentration given being calculated on lability. In the case of chromatographically pure preparations no special mention is made in the text.

Activity determination was carried out in two ways.

1. The first method was a slightly modified procedure of Liubimova and Engelgardt [15]. Solutions used: a) myosin, usually 0.31 mg/ml, in 0.5 M KCl, b) 0.05 M $CaCl_2$, c) borate buffer (Clarke), pH 9.0, d) ATP solution in buffer, pH adjusted to 9.0 on the glass electrode. The order of addition of the various components was

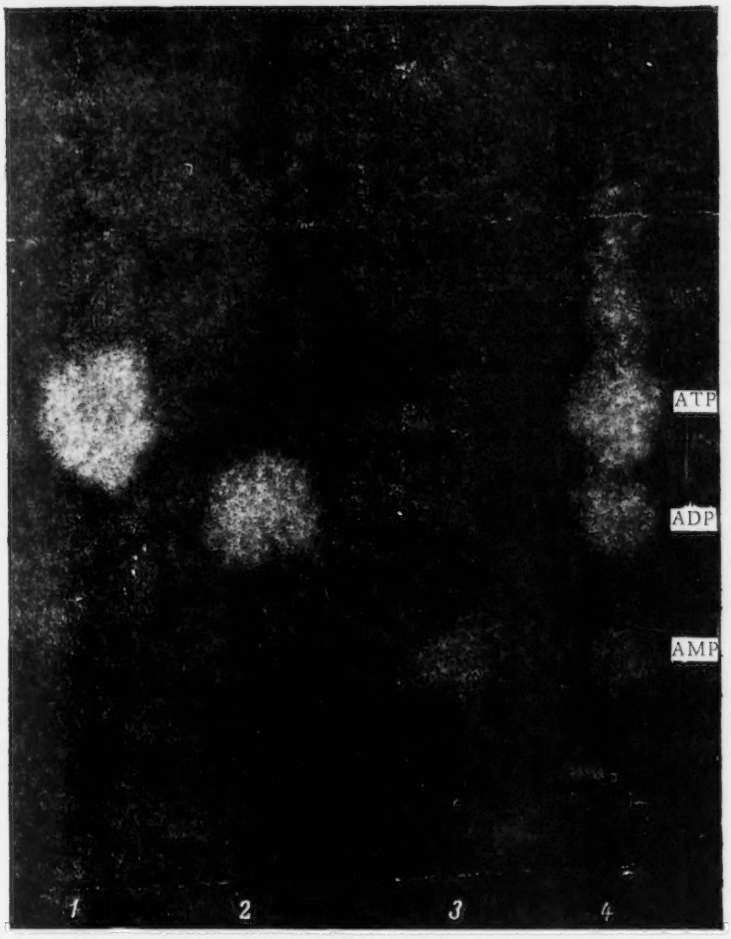

Fig. 3. Chromatography of column-purified ATP (1) and ADP (2) preparations: Markers: 3) AMP; 4) crude ATP preparation. Each test contained 100μg of the nucleotide. Solvent: n-propanol–ammonia–water (60 : 30 : 10); descending chromatography; 48 hrs, room temperature.

such that myosin was added last. The composition of the reaction was as follows: myosin 0.3 ml, $CaCl_2$ 0.1 ml, ATP 0.6 ml. Mixtures were incubated for 5 min at 37°. Final KCl concentration was 0.175 M. The reaction was stopped by the addition of 1.0 ml of 10% trichloroacetic acid. Phosphorus was determined according to Fiske-Subbarow. All results were expressed as μg P/ml.

2. Determinations of higher accuracy were made possible by the construction of an apparatus which allowed the exact determination of the starting and finishing time of the reaction and thus the measurement of reaction rates over shorter time intervals. The reaction was again stopped by the addition of trichloroacetic acid to the tube. The relative concentrations of the reaction components was the same as described in the first method above. Mixtures were incubated at 30°. This method was employed in the determination of initial reaction rates.

EXPERIMENTAL RESULTS

Multiple experiments, using both pure and crude ATP preparations, demonstrated the presence of a definite substrate inhibition effect on myosin ATPase activity.

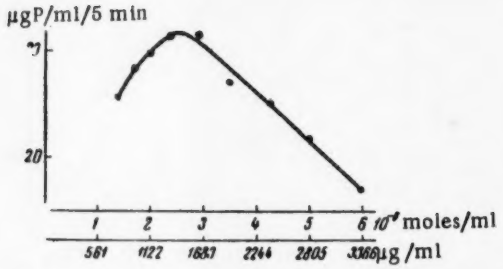

Fig. 4. Substrate inhibition of myosin ATPase activity. Incubation – 5 min at 37°; pH 9.0. Final myosin concentration 0.145 mg/ml throughout (Q_p = 1870). $CaCl_2$ – 0.005 M.

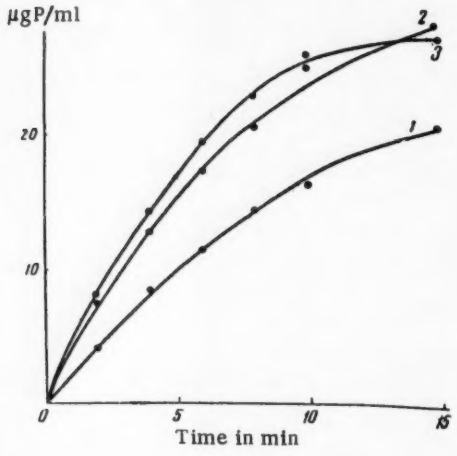

Fig. 5. The effect of substrate concentration on the dephosphorylation of ATP by myosin ATPase. Temperature 37°; pH 9.0; myosin concentration 0.093 mg/ml throughout; $CaCl_2$ – 0.005 M. Crude ATP preparation containing 83% ATP (as myosin attackable) and 15% ADP (by P_{10}). ATP concentrations: 1) $4.9 \cdot 10^{-3}$ M; 2) $2.4 \cdot 10^{-3}$ M; 3) $1.2 \cdot 10^{-3}$ M. ADP admixture: 1) $0.9 \cdot 10^{-3}$ M; 2) $0.44 \cdot 10^{-3}$ M; 3) $0.2 \cdot 10^{-3}$ M.

The results of one representative experiment are shown in Fig. 4.

At a myosin concentration of 0.145 mg/ml, and with ATP concentration ranging from 1.4 to $6.0 \cdot 10^{-3}$ M, it was possible to demonstrate the optimal ATP concentration – $2.5 \cdot 10^{-3}$ M – at which myosin ATPase reached its maximal reaction rate (31.4 μg P). Substrate inhibition was observed when ATP concentration was increased beyond this limit (hyperoptimal concentration). Only 17 μg P/ml/5 min was split at a ATP concentration $6.0 \cdot 10^{-3}$ M, that is, 54% of the maximal value. It appears that above optimal concentrations of ATP the reaction velocity was linear (inversely) with ATP concentration. On the left-hand side of the maximum, in the direction of decreasing ATP concentrations, the reaction velocity dropped rapidly, both as a result of decreasing initial velocities, and because of a relative substrate deficiency, since ATP hydrolysis at 37° proceeds with considerable speed and is well advanced at the end of the 5 min period.

The initial reaction velocities of ATPase also underwent characteristic changes with changing substrate concentration.

The progress of ATP hydrolysis by myosin ATPase at various ATP concentrations is shown in Fig. 5.

In agreement with the above-mentioned, the highest initial velocity was observed with the lowest of the three ATP concentrations tested, namely $1.2 \cdot 10^{-3}$ M; lower velocities were obtained with an ATP concentration of $2.4 \cdot 10^{-3}$ M, and lower still with $4.9 \cdot 10^{-3}$ M. In the subsequent period of the reaction (the reaction, of which the initial 15 min are shown on Fig. 5, was carried out for 30 min) the curve in Experiment 3 rapidly reached its limit as a result of substrate exhaustion, while a fairly energetic hydrolysis could still be observed in Experiment 2 at 30 min; in Experiment 1 the velocity of ATP dephosphorylation decreased rapidly.

The effect of ADP on the activity of myosin ATPase was investigated in many experiments and its inhibitory action was established.

The results of one representative experiment are shown in Fig. 6.

It will be seen from the graph that, with equimolar concentrations of ATP and ADP ($2 : 2 \cdot 10^{-3}$ M), the activity of myosin ATPase hardly reached $^1/_5$ of its original value. The inhibitory effect of ADP was still quite apparent when the ratio was lowered to 1 : 8 (activity observed – 60% of original).

The inhibitory effect of ADP on myosin ATPase was not removed by the exclusion of Ca from the system, although the degree of inhibition was considerably less pronounced. Thus, in one of the experiments, in the presence of Ca the amount of P split off was 14.1 μg/ml/5 min in the absence of ADP and 5.0 μg/ml/5 min in its presence; when Ca was excluded from the reaction mixture the respective values were 5.0 and 3.2 μg P/ml/5 min.

The inhibition of ATPase activity by ADP was also observed at pH 6.4 (trishydroxymethylaminomethane buffer – tris), as shown in Fig. 7.

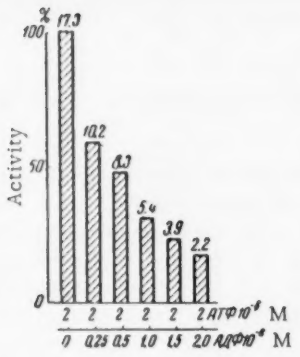

Fig. 6. The inhibition of myosin ATPase activity by ADP. Incubation – 5 min at 37°, pH 9.0. Final myosin concentration, 0.093 mg/ml; Q_p = 1540. $CaCl_2$ – 0.005 M. The figures above the columns refer to the absolute activity in μg P/ml/5 min.

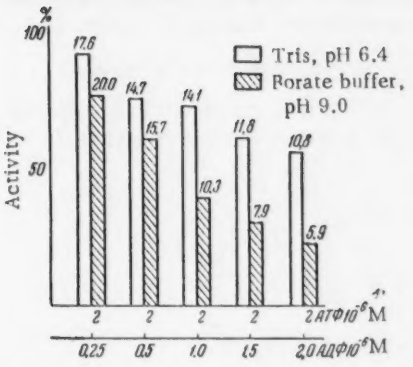

Fig. 7. The effect of pH on the inhibition of myosin ATPase by adenosine diphosphate. Incubation – 5 min at 37°. Final myosin concentration 0.13 mg/ml; $CaCl_2$ – 0.005 M. Initial activities at pH 9.0 – 25.5 μg P/ml/5 min. Corresponding activities in μg P/ml/5 min are shown above the columns.

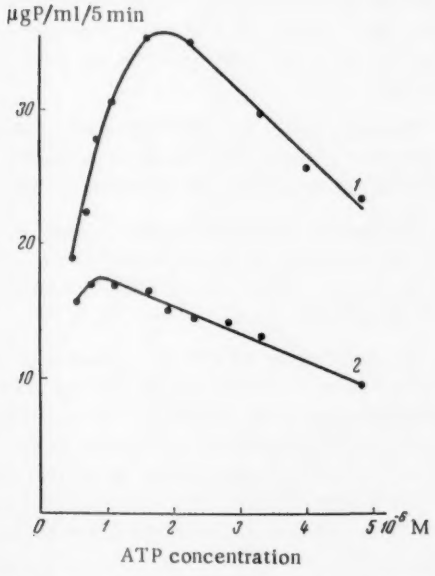

Fig. 8. Dependence of substrate inhibition of myosin ATPase activity on enzyme concentration. Incubation – 5 min at 37°; pH 9.0. Final myosin concentration (Q_p = = 1620): 1) 0.186 mg/ml; 2) 0.093 mg/ml; $CaCl_2$ – 0.005 M. Crude ATP preparation, myosin attackability 80%.

TABLE 1

Experiment no.	μg P/ml/5 min	%
1	28.5	100
2	22.2	78
3	21.6	76

The activity of myosin ATPase at pH 6.4 was somewhat lower than at pH 9.0 (in the given case – 74% of the latter). The inhibition by ADP was quite pronounced, though comparatively lower than the inhibition at pH 9.0.

The present results on the effect of Ca and the pH of the medium are in good agreement with those of Green and Mommaerts [6]; according to these authors, the exclusion of Ca from the reaction mixture lowers the affinity of ATP for the enzyme, as does the decrease in pH to 6.4. Accordingly, the K_i values for ADP, calculated by Green and Mommaerts, increase.

Experiments were also carried out with the aim of demonstrating that the use of crude ATP preparations in the determination of activities of myosin ATPase leads to artificially low results, due mainly to the presence of ADP in the preparation. The experiment consisted in the determination of myosin ATPase activity, using a crude ATP preparation containing, according to chromatographic analysis, 75% ATP and 16% ADP; the results were expressed as μg P/ml/5 min at 37°. The experiment was then repeated using an artificially prepared mixture consisting of ATP and ADP in the same proportion as the crude preparation.

Experimental conditions: temperature 37°, pH 9.0; incubation period 5 min. Final myosin concentration 0.13 mg/ml throughout; $CaCl_2$ – 0.005 M. Borate buffer, pH 9.0. Substrate: Experiment 1 – ATP, chromatographically pure, 1.8 μmole/ml, Experiment 2 – the same + 0.5 μmole/ml of chromatographically pure ADP;

Experiment 3 – crude ATP preparation containing 75% ATP and 16% ADP; concentrations of ATP and ADP in the reaction mixture – 2.15 and 0.46 μ moles /ml, respectively.

The results are shown in Table 1.

The activity values obtained in the case of the crude preparation and in the case of the mixture of purified ATP and ADP were found to be identical.

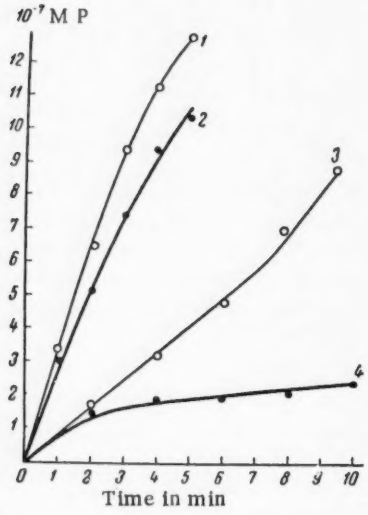

Fig. 9. The effect of myosin concentration on the inhibition of myosin ATPase by ADP. Temperature 30°, pH 9.0, ATP – $3 \cdot 10^{-3}$ M throughout. ADP: 2 and 4) $1.5 \cdot 10^{-3}$ M. Myosin : 1 and 2) 0.27 mg/ml, 3 and 4) 0.09 mg/ml; $CaCl_2$ – 0.005 M.

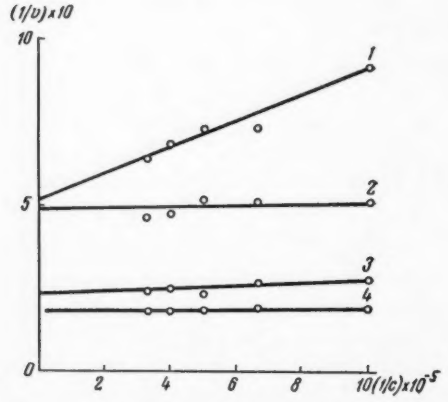

Fig. 10. The character of the inhibition of myosin ATPase by adenosine diphosphate. Temperature 30°, pH 9.0. ATP concentrations throughout 3.0-, 2.5-, 2.0-, 1.5-, $1.0 \cdot 10^{-3}$ M. ADP concentrations: 1 and 3, $1.0 \cdot 10^{-3}$ M. Myosin concentration: 1 and 2) 0.093 mg/ml; 3 and 4) 0.186 mg/ml. Abscissa – inverse values of molar ATP concentrations. Ordinate – inverse values of reaction velocities in μ g P per min.

It appears that the presence of ADP in the reaction mixture is of some importance in the substrate inhibition effect. This can be seen from a comparison of Figs. 4 and 8. In the first case (Fig. 4), chromatographically pure ATP and myosin at a final concentration of 0.145 mg/ml were employed; maximal reaction velocity was reached at an ATP concentration $2.5 \cdot 10^{-3}$ M (optimal concentration). In the experiment represented by Fig. 8 the ATP used was a crude preparation and the actual ATP content was calculated from myosin attackability data. The myosin concentration in a number of tests (curve 1) was higher than in the first case (0.186 mg/ml), but the optimal ATP concentration in this case fell to $2.0 \cdot 10^{-3}$ M. Insofar as such relationship held for a number of other experiments, it may be inferred that when crude ATP preparations were used, the decrease in optimal concentration was due to their ADP content.

The experiments described below were concerned with the effect of myosin ATPase concentration on the substrate inhibition and on ADP inhibition effects.

The optimal ATP concentration depends on the enzyme concentration in the incubation mixture. The results of a representative experiment with varying enzyme concentrations are shown in Fig. 8.

The ATP concentration in the present case was between 0.5 and $5.0 \cdot 10^{-3}$ M, and myosin was used in two concentrations – 0.186 and 0.093 mg/ml. The two curves obtained were similar in their shape to the curve shown in Fig. 4. The maxima of the two curves differ not only in height (which could be expected as a result of the different enzyme concentrations), but are also horizontally shifted relative to each other – the lower optimal ATP concentration corresponding to the lower enzyme concentration. In the experiment quoted the halving of the enzyme concentration caused a shift of the optimal ATP concentration from 1.8 to $0.9 \cdot 10^{-3}$ M – also half. Incidentally, such a coincidence was not observed in all cases. Myosin activity decreased with storage, and consequently there also occurred a decrease in the optimal ATP concentration observed, that is a shift took place in conformity with the enzyme concentration.

It was further found that the inhibitory effect of ADP was also dependent on the enzyme concentration. With the enzyme concentration at 0.093 mg/ml (Q_p of myosin 1800-2000), the inhibitory effect of ADP was

invariably observed, although the degree of inhibition varied in individual experiments. A 2-3-fold increase of myosin concentration sharply decreased the inhibitory effect of ADP on myosin ATPase activity. This is demonstrated in Fig. 9; with equal ATP to ADP ratios, here illustrated in curves 1 and 2, the myosin concentration was increased up to three times in a series of tests – from 0.9 to 0.27 mg/ml. The result was a distinct fall of the inhibitory effect of ADP. The effect of myosin concentration on the inhibitory action of ADP was not confined to the above phenomenon. The inhibitory effect was altered not only quantitatively, but also qualitatively, apparently as a result of an altered mode of interaction between ADP and myosin ATPase.

Presented in Fig. 10 are the results of experiments with one myosin preparation with equal, and exactly determined, quantities of chromatographically pure ATP and ADP.

The apparatus mentioned in the "Methods" section was used in the determination of the initial hydrolysis rates. The graphical representation of data was according to Lineweaver and Burk [16] – the inverse of the reaction velocities being plotted against the inverse of the substrate concentrations. Myosin concentration at 0.093 mg/ml was employed in the two cases represented by curves 1 and 2, corresponding to incubations carried out in the presence and absence of ADP, respectively. It is quite obvious that the phenomenon observed is competitive inhibition, or very closely related to it. Curves 3 and 4 represent a set of conditions identical to 1 and 2, respectively, with the exception that the myosin concentration employed was 0.186 mg/ml. Here not only the absolute degree of inhibition by ADP was decreased, as had already been mentioned above, but also the character of the effect was altered – it became noncompetitive.

DISCUSSION OF RESULTS

On the basis of the results presented above, the substrate inhibition effect on myosin ATPase may be taken as demonstrated beyond doubt. The results described above confirmed the earlier findings of Hasselbach [4] who showed that ATPase activity of myosin and actomyosin solutions decreased linearly when the ATP concentration was increased from $0.4 \cdot 10^{-3}$ to $7.0 \cdot 10^{-3}$ M.

The doubts expressed by Green and Mommaerts [6] with regard to substrate inhibition, appear to the present authors to have been without foundation. In the experiments of Green and Mommaerts [6] the ATP concentrations ranged between 0.08 and $0.33 \cdot 10^{-3}$ M, and myosin concentration between 0.008 and 0.16 mg/ml, consequently substrate inhibition could hardly have been observed under their conditions. The authors themselves mentioned the fact that the ATP concentrations employed by them were 10 times lower than those employed by Hasselbach.

Substrate inhibition can, apparently, take place even in more complicated systems than myosin solutions; one case, at least, has been described by Perry and Grey in experiments with muscular fibril models [17]. Using isolated myofibrils, these authors showed that, at μ = 0.06 and pH 6.9-7.6, ATP concentrations, exceeding $4 \cdot 10^{-3}$ M, produced an inhibition of the ATPase activity of the myofibrils. The existence of optimal ATP concentration values, at which the activity of myosin ATPase reached maximum under a given set of conditions, is related to the substrate inhibition effect and is its natural result. Of interest is the very narrow range of optimal ATP concentrations, although in this respect myosin ATPase is not an exception.

A logical inference, arising in this connection, is that there may exist a definite and constant ratio of myosin and ATP concentrations in the zone of maximal reaction velocity. Relevant calculations were therefore made, in which the optimal molar concentration of ATP was related to the myosin concentration, also expressed in moles. Here the molecular weight of myosin was taken as 900,000 [2], and the mean activity of the preparations as Q_p = = 1800-2000. Whenever the activity of any myosin preparation diverged significantly from this value, a correction coefficient was introduced into the calculations, since it could be assumed that in this case the important factor was not the protein concentration, but the concentration of its active centers, which would be equivalent in activity under equal experimental conditions.

The calculations showed that the expected ratio actually existed and that its value was $1.0\text{-}1.5 \cdot 10^{-4}$ for myosin with an activity of Q_p = about 1800.

The present results confirmed those of Kalckar [5] and of Green and Mommaerts [6] on the inhibitory effect of ADP on myosin ATPase activity. It should also be added that many experimental conditions in the present experiments (KCl and $CaCl_2$ concentrations, pH, ADP concentration) were identical with those employed by Green and Mommaerts.

As already mentioned above, Blum [7] considers the inhibitory effect of ADP insignificant.

At ATP concentrations $2.14 \cdot 10^{-4}$ M, and ADP concentrations $2.2 \cdot 10^{-3}$ M (the latter concentration being ten times the former), the ATPase activity was found by Blum to be 71.4% of the activity of control tests, containing no ADP. It should be added that the author carried out all determinations at high KCl concentrations (0.6 M) and pH 7.0 with tris buffer. Protein concentration was not specified in the particular tests, but the concentration used throughout the work ranged from 0.04 to 0.12 mg protein/ml.

The discrepancy between the results of Blum and those in the present work may be explained by the fact that, in the work described by Blum, the significant factors may have been the high KCl concentration (it inhibits ATPase activity) and the pH of the medium employed (the affinity of ADP for the enzyme is lowered at neutral pH).

The data quoted below from the literature indicate the high affinity of ADP for myosin ATPase, comparable with that of ATP.

According to the calculations of Green and Mommaerts, K_i for ADP ranged, depending on experimental conditions, from $1.4 \cdot 10^{-4}$ M to $1.6 \cdot 10^{-5}$ M. This value is fairly close to the K_m value which, according to Tonomura [18], equals $1.5 \cdot 10^{-4}$ for rabbit muscle actomyosin at 12°, pH 6.8-7.0, $1 \cdot 10^{-2}$ M Ca^{++} and 0.15 M KCl. A similar value for K_m, $1.6 \cdot 10^{-4}$ M, quoted by Watanabi, Yagi and Tonomura [19] was obtained under experimental conditions closely resembling those quoted above.

It is also of some interest to note the interesting results of some experiments with the ATPase of the avian erythromyeloblastic leukemia [20, 21], which have demonstrated that the K_m for this enzyme was close to that of myosin, the quoted value being $0.73 \cdot 10^{-4}$ M. The existence of competitive inhibition of the enzyme by ADP was established by these workers and the value of K_i for ADP calculated and found to be $1.85 \cdot 10^{-5}$ M.

The most important point in the present work is, in the author's opinion, the finding on the importance of the myosin concentration for the demonstration of the substrate inhibition effect, as well as of the competitive inhibition by ADP. As far as the author is aware, this factor has not received to date due attention, although its importance in the kinetics of myosin ATPase is quite real.

The shift of the critical ATP concentration to higher values with increasing myosin concentrations is easily understood, if the existence of the definite ratio between ATP and myosin, as mentioned above, be taken into consideration. Under those conditions each change in myosin concentration should be followed by a corresponding shift in the optimal ATP concentration, which has actually been observed.

The experiments of Perry and Grey mentioned earlier prompt the speculation that the substrate inhibition observed in vitro could also take place in vivo, but the concentration ratio of myosin and ATP in muscle is such that the conditions for its realization are absent.

Indeed, if the myosin content of muscle under physiological conditions be taken as 20%, and that of ATP 0.25-0.4%, then the molar ATP/myosin ratio in situ would be $0.25\text{-}0.5 \cdot 10^2$, that is, two orders of magnitude below the lowest value at which substrate inhibition can be demonstrated in vitro. Apart from this, there always remains an element of doubt as to whether these mean concentrations of ATP and myosin, quoted above, correspond to the local concentrations within the muscle fibers, on the sites of ATPase action.

According to Perry and Grey [17], the concentration of the metal activator, in this case Mg^{++}, is of considerable importance in the demonstration of substrate inhibition of ATPase activity. Substrate inhibition, in fact, was observed as soon as the ATP concentration reached that of the metal ion. In contradistinction, the inhibition was completely reversed by the addition of Ca. This shows that the substrate inhibition phenomenon may be a complicated one, and that any simple interpretation, based on a concept of substrate binding to the center of the enzyme devoid of activity, may be quite erroneous. The ATP molecule is known to have a high affinity for metal ions [22, 23] and it is possible that, in the presence of excessive concentrations of ATP, distributions of mono- and divalent ions, unfavorable to ATPase activity, may occur. This aspect, however, was not included in the present investigation.

The influence of the myosin ATPase concentration on the inhibitory effect of ADP is of great interest. It was seen from the results that this effect consisted in lowering the level of inhibitory activity of ADP, as well as abolishing the competitive nature of the inhibition. It is difficult to offer at present an explanation of this phenomenon,

but it seems clear that the conventional kinetic equations, based on the Michaelis-Menten theory, are incapable of solving this problem. The possibility is not excluded that in this case the concentrations of myosin, ATP and ADP are not the only operative factors. $CaCl_2$ concentration, which was invariably 0.005 M in all cases, was not taken into consideration. It is possible that the inhibitory effect depends on this divalent ion. This is also suggested by the work of Perry and Grey [17] and of Green and Mommaerts [6], according to whom the value of K_i for ADP depends $CaCl_2$ and $MgCl_2$ concentrations.

The inhibitory effect of ADP on myosin ATPase could hardly be expected to operate under physiological conditions, in view of the various powerful aerobic and anaerobic mechanisms in operation which would tend to eliminate it. The high protein concentration, presumably existing in the myofibril, provides another factor which ensures the high level of enzymatic activity.

The effect of protein concentration in the ADP inhibition phenomenon, established in the present work, explains the nearly complete splitting of the ATP in the enzymatic ADP preparation from ATP using myosin ATPase according to the method of Liubimova and Pevzner [13]. It is seen clearly from Fig. 2 that, when ATP is treated with a sufficiently high concentration of myosin, the amount of residual uncleaved ATP was negligible. This was almost undoubtedly due to the employment of high myosin concentrations (about 1 mg protein/ml) in the preparation of ADP.

SUMMARY

The existence of a definite substrate inhibition of myosin ATPase has been demonstrated.

ADP was shown to be inhibitory to myosin ATPase activity. This effect was shown to be competitive under certain conditions.

Enzyme concentration affected both the substrate inhibition and the inhibition by ADP. With increased enzyme concentrations in the reaction mixture the optimal concentration levels of ATP were also increased, so that substrate inhibition under those conditions was observed at higher ATP concentrations than was the case with the lower enzyme concentrations.

Increase in enzyme concentration also lowered and abolished the inhibitory effect of ADP, as well as removing its competitive property.

The mechanism by which the enzyme concentration affects the inhibitory effect of ADP on ATPase cannot be explained at present: the possibility that divalent ions (Ca) take part in this phenomenon was discussed.

The effect of myosin ATPase concentration on the inhibitory activity of ADP mentioned above is of considerable importance in in vitro experiments and, possibly, also in the living cell.

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* Original Russian pagination. See C. B. translation.

Symbols and Abbreviations Used in the Journal

1. Units

Kilogram	kg	Normality	N
Gram	g	Millimole	
Milligram	mg	(10^{-3} mole)	mmole
Microgram	γg	Micromole	
Liter	l	(10^{-6} mole)	μ mole
Cubic centimeter		Atmosphere	atm.
(milliliter)	ml	Kilocalorie	kcal.
Cubic millimeter		Calorie	cal.
(microliter)	μ l	Volt	v
Meter	m	Ampere	amp.
Millimeter	mm	Watt	w
Micron (0.001 mm)	μ	Curie	C
Millimicron		Millicurie	mC
(0.001 μ)	mμ	Microcurie	μ C
Molarity	M	Roentgen	r

2. Abbreviations for Biochemical Substances

ADP	adenosinediphosphate	IMP	inosinemonophosphate (Inosinic acid)
ACTH	adrenocorticotropic hormone	ITP	inosinetriphosphate
		K_m	Michaelis constant
AMP	adenosinemonophosphate (adenylic acid)	CoA	coenzyme A
		RNA	ribonucleic acid
		RNase	ribonuclease
ATP	adenosinetriphosphate	TPN	triphosphopyridine nucleotide
		TPN·2H	reduced form of triphosphopyridine nucleotide
ATPase	adenosinetriphosphatase		
GDP	guanosinediphosphate	UDP	uridinediphosphate
		UMP	uridinemonophsphate
GMP	guanosinemonophosphate (guanylic acid)	UTP	uridinetriphosphate
		FAD	flavin adenine dinucleotide
GTH	gonadotropic hormones	FMN	flavin mononucleotide
GTP	guanosinetriphosphate	TAC	tricarboxylic acid cycle
		F-1,6-P	fructose-1,6-diphosphate
DNA	desoxyribonucleic acid	F-1-P	fructose-1-phosphate
		F-6-P	fructose-6-phosphate
DNase	desoxyribonuclease	G-1-P	glucose-1-phosphate
DPN	diphosphopyridine nucleotide	G-6-P	glucose-6-phosphate
DPN·2H	reduced form of diphosphopyridine nucleotide	TCA	trichloroacetic acid
		tris	tris(hydroxymethylamino) methane
IDP	inosinediphosphate	MB	methylene blue

3. The following abbreviations are used for amino acid residues in polypeptides and proteins:

al.	alanine	lys.	lysine
arg.	arginine	meth.	methionine
asp-NH_2	asparagine	norl.	norleucine
asp.	aspartic acid	hydroxyprol.	hydroxyproline
val.	valine	prol.	proline

his.	histidine	ser.	serine
glyc.	glycine	tyr.	tyrosine
glut-NH_2	glutamine	thre.	threonine
glut.	glutamic acid	tryp.	tryptophane
isol.	isoleucine	phal.	phenylalanine
leuc.	leucine	cystine	cystine
		cysteine	cysteine

The configuration of optically active compounds is indicated by the symbols D and L; racemates are denoted by DL.

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ABBREVIATIONS MOST FREQUENTLY ENCOUNTERED IN RUSSIAN BIO-SCIENCES LITERATURE

Abbreviation (Transliterated)	Significance
AMN SSSR	Academy of Medical Sciences, USSR
AN SSSR	Academy of Sciences, USSR
BIN	Biological Institute, Botanical Institute
FTI	Institute of Physiotherapy
GONTI	State United Sci-Tech Press
GOST	All Union State Standard
GRRRI	State Roentgenology, Radiology, and Cancer Institute
GTTI	State Technical and Theoretical Literature Press
GU	State University
I Kh N	Scientific Research Institute of Surgical Neuropathology
IL (IIL)	Foreign Literature Press
IONKh	Inst. Gen. and Inorganic Chemistry (N. S. Kurnakov)
IP	Soil Science Inst. (Acad. Sci. USSR)
ISN (Izd. Sov. Nauk)	Soviet Science Press
Izd.	Press
LEM	Laboratory for experimental morphogenesis
LENDVI	Leningrad Inst. of Dermatology and Venereology
LEO	Laboratory of Experimental Zoology
LIKhT	Leningrad Surgical Institute for Tuberculosis and Bone and Joint Diseases
LIPZ	Leningrad Inst. for Study of Occupational Diseases
LIPK	Leningrad Blood Transfusion Institute
Medgiz	State Medical Literature Press
MOPISh	Moscow Society of Apiculture and Sericulture
MVI	Moscow Veterinary Institute
MZdrav	Ministry of Health
MZI	Moscow Zootechnical Institute
LOKhO	Leningrad Society of Orthopedic Surgeons
NIIZ	Scientific Research Institute of Zoology
NINKhI	Scientific Research Institute of Neurosurgery
NIU	Scientific Institute for Fertilizers
NIUIF	Scientific Research Institute of Fertilizers and Insecticides
NIVI	Veterinary Scientific Research Institute
ONTI	United Sci.-Tech. Press
OTI	Division of Technical Information
RBO	Russian Botanical Society
ROP	Russian Society of Pathologists
SANIIRI	Central Asia Scientific Research Institute of Irrigation
SANIISh	Central Asia Scientific Research Institute of Sericulture
TsNII	All-Union Central Scientific Research Institute
TsNTL	Central Scientific and Technical Laboratory
VASKhNIL	All-Union Academy of Agricultural Sciences
VIG	All-Union Institute of Helminthology
VIEM	All-Union Institute of Experimental Medicine
VIR	All-Union Institute of Plant Cultivation
VIUAA	All-Union Institute of Fertilizers, Soil Science, and Agricultural Engineering
VIZR	All-Union Institute of Medical and Pharmaceutical Herbs
VNIRO	All-Union Scientific Institute of Fishing and Oceanography
ZIN	Zoological Inst. (Acad. Sci. USSR)

Note: Abbreviations not on this list and not explained in the translation have been transliterated, no further information about their significance being available to us. - Publisher.

RUSSIAN JOURNALS FREQUENTLY CITED

[Biological Sciences]

Abbreviation*	Journal*	Translation
Agrobiol.	Agrobiologiia	Agrobiology
Akusherstvo i Ginekol.	Akusherstvo i Ginekologiia	Obstetrics and Gynecology
Antibiotiki	Antibiotiki	Antibiotics
Aptechnoe Delo	Aptechnoe Delo	Pharmaceutical Transactions
Arkh. Anat. Gistol. i Embriol.	Arkhiv Anatomii Gistologii i Embriologii	Archives of Anatomy, Histology, and Embryology
Arkh. Biol. Nauk SSSR	Arkhiv Biologicheskikh Nauk SSSR	Archives of Biological Science USSR
Arkh. Patol.	Arkhiv Patologii	Archives of Pathology
Biofizika	Biofizika	Biophysics
Biokhimiia	Biokhimiia	Biochemistry
Biokhim. Plodov i Ovoshchei	Biokhimiia Plodov i Ovoshchei	Biochemistry of Fruits and Vegetables
Bot. Zhur.	Botanicheskii Zhurnal	Journal of Botany
Biull. Ekspti. Biol. i Med.	Biulleten Eksperimentalnoi Biologii i Meditsiny	Bulletin of Experimental Biology and Medicine
Biull. Moskov. Obshchestva Ispytatelei Prirody, Otdel Biol.	Biulleten Moskovskogo Obshchestva Ispytatelei Prirody, Otdel Biologicheskii	Bulletin of the Moscow Naturalists Society, Division of Biology
Doklady Akad. Nauk SSSR	Doklady Akademii Nauk SSSR	Proceedings of the Academy of Sciences USSR
Ekspti. Khirurg.	Eksperimentalnaia Khirurgiia	Experimental Surgery
Farmakol. i Toksikol.	Farmakologiia i Toksikologiia	Pharmacology and Toxicology
Farmatsiia	Farmatsiia	Pharmacy
Fiziol. Rastenii	Fiziologiia Rastenii	Plant Physiology
Fiziol. Zhur. SSSR	Fiziologicheskii Zhurnal SSSR im. I. M. Sechenova	I. M. Sechenova Physiology Journal USSR
Gigiena i Sanit.	Gigiena i Sanitariia	Hygiene and Sanitation
Izvest. Akad. Nauk SSSR, Ser. Biol.	Izvestiia Akademii Nauk SSSR, Seriia Biologicheskaia	Bulletin of the Academy of Sciences USSR, Biology Series
Izvest. Tikhookeanskogo N. I. Inst. Rybnogo Khoz. i Okeanog.	Investiia Tikhookeanskogo N. I. Instituta Rybnogo Khoziaistva i Okeanografii	Bulletin of the Pacific Ocean Scientific Institute of Fisheries and Oceanography
Khirurgiia	Khirurgiia	Surgery
Klin. Med.	Klinicheskaia Meditsina	Clinical Medicine
Lab. Delo	Laboratornoe Delo(po Voprosam Meditsiny)	Laboratory Work (on Medical Problems)
Med. Parazitol.	Meditsinskaia Parazitologiia i Parazitarnye Bolezni	Medical Parasitology and Parasitic Diseases
Med. Radiol.	Meditsinskaia Radiologiia	Medical Radiology
Med. Zhur. Ukrain.	Medichnii Zhurnal Ukrainskii	Ukrainian Medical Journal
Mikrobiologiia	Mikrobiologiia	Microbiology
Mikrobiol. Zhur.	Mikrobiologichnii Zhurnal	Microbiology Journal
Nevropatol., Psikhiat. i Psikhogig.	Nevropatologiia, Psikhiatriia i Psikhogigiena	Neuropathology, Psychiatry and Psychohygiene
Ortoped., Travmatol. i Protez.	Ortopediia, Travmatologiia i Protezirovanie	Orthopedics, Traumatology and Prosthetics
Parazitol. Sbornik	Parazitologicheskii Sbornik	Parasitology Collection
Pediatriia	Pediatriia	Pediatrics
Pochvovedenie	Pochvovedenie	Soil Science
Priroda	Priroda	Nature
Problemy Endokrinol. i Gormonoterap.	Problemy Endokrinologii i Gormonoterapii	Problems of Endocrinology and Hormone Therapy
Problemy Gematol.	Problemy Gematologii i Perelivaniia Krovi	Problems of Hematology and Blood Transfusion
Problemy Tuberk.	Problemy Tuberkuleza	Problems of Tuberculosis
Sovet. Med.	Sovetskaia Meditsina	Soviet Medicine
Sovet. Vrachebny Zhur.	Sovetskii Vrachebnyi Zhurnal	Soviet Physicians Journals
Stomatologiia	Stomatologiia	Stomatology
Terap. Arkh.	Terapevticheskii Arkhiv	Therapeutic Archives
Trudy Gelmint. Lab.	Trudy Gelmintologicheskoi Laboratoriia	Transactions of the Helminthology Laboratory
Trudy Inst. Genet.	Trudy Instituta Genetiki	Transactions of the Institute of Genetics

* LIBRARY OF CONGRESS TRANSLITERATION SYSTEM.

(continued)

(continued)

Abbreviation	Journal	Translation
Trudy Inst. Gidrobiol.	Trudy Instituta Gidrobiologiia	Transactions of the Institute of Hydrobiology
Trudy Inst. Mikrobiol.	Trudy Instituta Mikrobiologiia	Transactions of the Institute of Microbiology
Trudy Inst. Okean.	Trudy Instituta Okeanologiia, Akademii Nauk SSSR	Transactions of the Institute of Oceanology, Academy of Sciences, USSR
Trudy Leningrad Obshchestva Estestvoisp.	Trudy Leningrad Obshchestva Estestvoispytatelei	Transactions of the Leningrad Society of Naturalists
Trudy Vsesoiuz. Gidrobiol. Obshchestva	Trudy Vsesoiuznogo Gidrobiologicheskogo Obshchestva	Transactions of the All-Union Hydrobiological Society
Trudy Vsesoiuz. Inst. Eksptl. Med.	Trudy Vsesoiuznogo Instituta Eksperimentalnoi Meditsiny	Transactions of the All-Union Institute of Experimental Medicine
Ukrain. Biokhim. Zhur.	Ukrainskii Biokhimichnii Zhurnal	Ukrainian Biochemical Journal
Urologiia	Urologiia	Urology
Uspekhi Biokhimiia	Uspekhi Biokhimiia	Progress in Biochemistry
Uspekhi Sovremennoi Biol.	Uspekhi Sovremennoi Biologiia	Progress in Contemporary Biology
Vestnik Akad. Med. Nauk SSSR	Vestnik Akademii Meditsinskikh Nauk SSSR	Bulletin of the Academy of Medical Science USSR
Vestnik Khirurg. im. Grekova	Vestnik Khirurgii imeni Grekova	Grekov Bulletin of Surgery
Vestnik Leningrad. Univ. Ser. Biol.	Vestnik Leningradskogo Universiteta, Seriia Biologii	Journal of the Leningrad Univ., Biology Series
Vestnik Moskov. Univ., Ser. Biol. i Pochvov.	Vestnik Moskovskogo Universiteta, Seriia Biologii i Pochvovedeniia	Bulletin of the Moscow University, Biology and Soil Science Series
Vestnik Oftalmol.	Vestnik Oftalmologii	Bulletin of Ophthalmology
Vestnik Oto-rino-laringol.	Vestnik Oto-rino-laringologiia	Bulletin of Otorhinolaryngology
Vestnik Rentgenol. i Radiol.	Vestnik Rentgenologii i Radiologii	Bulletin of Roentgenology and Radiology
Vestnik Venerol. i Dermatol.	Vestnik Venerologii i Dermatologii	Bulletin of Venereology and Dermatology
Veterinariia	Veterinariia	Veterinary Science
Vinodelie i Vinogradarstvo	Vinodelie i Vinogradarstvo SSSR	Wine-Making and Viticulture
Voprosy Klin.	Voprosy Klinicheskie	Clinical Problems
Voprosy Med. Khim.	Voprosy Meditsinskoi Khimii	Problems of Medical Chemistry
Voprosy Med. Virusol.	Voprosy Meditsinskoi Virusologii	Problems of Medical Virology
Voprosy Neirokhirurg.	Voprosy Neirokhirugii	Problems of Neurosurgery
Voprosy Onkol.	Voprosy Onkologii	Problems of Oncology
Voprosy Pitaniia	Voprosy Pitaniia	Problems of Nutrition
Voprosy Psikhologii	Voprosy Psikhologii	Problems of Psychology
Voprosy Virusologii	Voprosy Virusologii	Problems of Virology
Vrachebnoe Delo	Vrachebnoe Delo	Medical Profession
Zav. Lab.	Zavodskaia Laboratoriia	Factory Laboratory
Zhur. Mikrobiol., Epidemiol. i Immunobiol.	Zhurnal Mikrobiologii, Epidemiologii i Immunobiologii	Journal of Microbiology, Epidemiology, and Immunobiology
Zhur. Nevropatol. i Psikhiat.	Zhurnal Nevropatologii i Psikhiatrii imeni S. S. Korsakov	S. S. Korsakov Journal of Neuropathology and Psychiatry
Zhur. Obshchei Biol.	Zhurnal Obshchei Biologiia	Journal of General Biology
Zhur. Vysshei Nerv. Deiatel.	Zhurnal Vysshei Nervnoi Deiatelnosti imeni I. P. Pavlova	I. P. Pavlov Journal of Higher Nervous Activity
Zool. Zhur.	Zoologicheskii Zhurnal	Journal of Zoology

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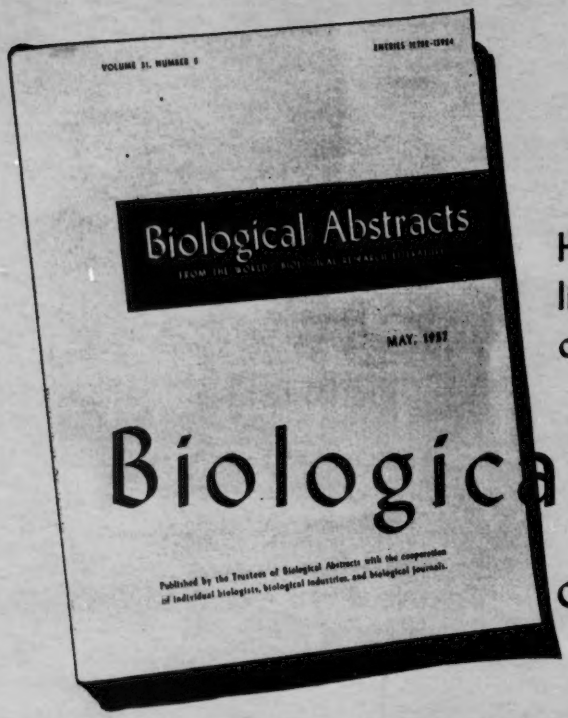
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